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Synthetic antiprotozoal thiazolide drug induced apoptosis in colorectal cancer cells: implications of IL-6/JAK2/STAT3 and p53/caspases-dependent signaling pathways based on molecular docking and in vitro study

Mohamed A. Tantawy[1,2,6] · Nagla A. El-Sherbeeny[3] · Nawal Helmi[4] · Reem Alazragi[4] · Neveen Salem[4,5] · Samah M. Elaidy[3]

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Abstract

Colorectal cancer (CRC) is a global pressing healthcare priority. Dysregulation of the IL6/JAK2/STAT3 and p53/caspase downstreaming pathways are significantly involved in the progression of CRC, and mainly affecting apoptosis. Discovery of new anti-cancer agents is laborious, time consuming, and costly with obvious socioeconomic burden. In the present study, we are proposing new molecular insights on the anti-proliferative and apoptotic therapeutic effects of nitazoxanide (NTZ) on CRC. NTZ is FDA-approved thiazolide antiparasitic agent, which has excellent safety and pharmacokinetic profiles. The molecular docking study revealed that NTZ has better binding affinity and docking score against JAK2 and BCL2 proteins compared to 5-Fluorouracil, which is the standard drug for treatment of CRC. The current in vitro work on a human HCT116 cell line displayed that NTZ had lower IC_{50} value (11.20 µM) than 5-flurouracil (23.78 µM), and NTZ induced a statistically significant down-regulation of IL6/JAK2/STAT3. NTZ also modulated significantly the p53/caspases-dependent signaling pathways, leading to enhancement of apoptosis and an increase of DNA fragmentation. Moreover, NTZ regulated the Bcl-2 gene family and promoted the loss of mitochondrial function which was depicted by release of cytochrome c (Cyt c), and caspase activation in apoptotic HCT116 cells. Additionally, NTZ was able to reduce the expression of VEGF in CRC cell line, which needs future thorough molecular investigations. In conclusion, our findings provided a novel evidence that NTZ could be a dual potential IL6/JAK2/STAT3 signaling inhibitor and p53/caspases-dependent pathway activator in CRC cell line. These potentials support further exploratory molecular researches targeting the therapeutic roles of NTZ in CRC; individually and simultaneously with current approved chemotherapeutic regimens.

Keywords Apoptosis · Colorectal cancer · Molecular docking · IL6/JAK2/STAT3 · p53/caspases · Nitazoxanide

✉ Mohamed A. Tantawy
Mohamed_tantawy@daad-alumni.de

✉ Samah M. Elaidy
samah_elaidi@med.suez.edu.eg

[1] Stem Cells Lab, Center of Excellence for Advanced Sciences, National Research Centre, Dokki, Giza 12622, Egypt

[2] Hormones Department, Medical Research Division, National Research Centre, Dokki, Giza 12622, Egypt

[3] Department of Clinical Pharmacology, Faculty of Medicine, Suez Canal University, Ismailia 41522, Egypt

[4] Department of Biochemistry, College of Science, University of Jeddah, Jeddah, Saudi Arabia

[5] Narcotics, Ergogenic Aids and Poisons Department, National Research Centre, Dokki, Giza 12622, Egypt

[6] Medical School of Hannover, Hannover, Germany

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Introduction

Worldwide, colorectal cancer (CRC) is ranked as the third most common cancer and the fourth in cancer-related mortality [1]. Treating CRC is usually multimodal including surgery, perioperative chemotherapy and -in certain cases- radiotherapy. Despite the advances in pharmacotherapy, drug resistance are challenging the efforts to minimize death from metastatic disease associated with CRC [2]. There is a compelling need to develop new anti-CRC drugs with better efficacy and safety than the existing ones.

Because drug discovery and development are demanding in terms of time and cost, a new strategy to advance new drugs faster is repurposing of available drugs to a new indication. This strategy speeds basic and preclinical research and provide candidates for clinical trials in much less time and cost. The thiazolide compound nitazoxanide (NTZ) is Food and Drug Administration (FDA)-approved antiparasitic drug with favorable pharmacokinetics and safety profiles (Fig. 1) [3, 4]. The drug is currently appreciated for its potential anti-cancer effects making it an ideal candidate for repurposing to this indication [5]. The anti-proliferative effect of NTZ was reported in ovarian, colon cancer and in glioblastoma [6–8].

Abnormalities of cellular signaling pathways occurs in almost all types of cancers [9]. It is crucial to understand how drugs modulate these pathways to change the trajectory of malignant cells from survival to committing suicide either by apoptosis or autophagy. Inflammation and activation of the IL-6/JAK/STAT3 pathway is thought to contribute to the pathogenesis of CRC [10]. Activated STAT3 is a transcription factor targeting genes expressing cyclin D1, c-myc, Bcl-xL, Mcl1 and vascular endothelial growth factor (VEGF). Induction of expression of these factors make this pathway critical in tumor differentiation, proliferation, and invasion. Therefore, inhibition of this pathway could have a role in preventing tumor growth by drugs [11].

The tumor suppressor gene *TP53* and its expressed protein P53 play a major role in keeping the cell genome intact by regulating cell cycle checkpoints, apoptosis, cell stress response, and inhibition of angiogenesis. In human CRC, studies reported overexpression and mutations of P53, which were associated with tumor progression and poor prognosis [12].

The known mechanism of action of NTZ is inhibition of pyruvate-ferredoxin oxidoreductase in protozoa and bacteria. This enzyme is essential for anaerobic metabolism in these organisms [13]. Recently, studies are indicating other possible mechanistic effects mediating its anti-cancer effects. NTZ was reported as an inhibitor of mitochondrial oxidative phosphorylation [7]. Another study found that NTZ was able to enhance endoplasmic reticulum stress and unfolded protein response [6]. NTZ decreased expression of the oncogene c-Myc in cancer cell lines, in particular CRC cells [5, 14]. The drug modulated AMPK/mTOR pathway resulting in increased apoptosis [7].

5-flurouracil (5-FU), is used as first-line chemotherapeutic in CRC drug regimens. It could be used alone with targeted therapy or combined with other cytotoxic drugs such as, with, oxaliplatin and/or irinotecan [15]. NTZ potentiated the in vivo anti-cancer effects of irinotecan in CRC [7].

The present study aimed to examine the in vitro antiproliferative and apoptotic effects of NTZ on a human CRC cell line (HCT116) alone or in combination with (5-FU). Moreover, the involvement of IL-6/JAK2/STAT3 and p53/caspases-dependent signaling pathways were studied.

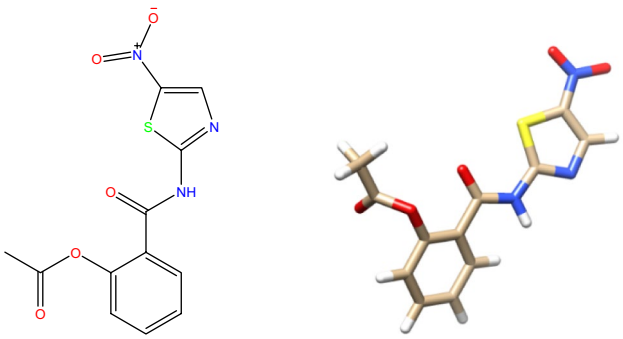

Fig. 1 2D and 3D structure of 2-((5-nitrothiazol-2-yl)carbamoyl) phenyl acetate (Nitazoxanide). 2D was generated using ChemDraw® Ultra program (version 8.0 April 23, 2003), while 3D structure was generated using UCSF Chimera (version 1.11.2)

Materials and methods

In silico study

The structure of the NTZ and 5-FU was modeled using the software of ChemDraw® Ultra program (version 8.0 April 23, 2003). The optimization and energy minimization of the structures were done by using the VEGAZZ software [16]. The Protein Data Bank (PDB) (www.rcsb.org) was used for obtaining the three-dimensional structures of the molecular target which were: Janus kinase 2 (JAK2) (PDB: 5AEP, https://www.rcsb.org/structure/5aep), and B-cell lymphoma-2 (BCL2) (PDB: 2O2F, https://www.rcsb.org/structure/2O2F). Preparation of the receptor has three steps according to our previous protocol [17]; first by removing of the heteroatoms like water and ions, second by addition of polar hydrogen, and finally the assignment of charge. The active sites were defined using grid boxes of appropriate sizes around the bound cocrystal ligands. The Autodock vina

[18] and Chimera for visualization [19] programs were used for docking study of the tested compounds.

Cell cultures

A Human colorectal carcinoma HCT116, was provided from the American Type Culture Collection (ATCC, USA) via VACERA Co. (Egypt) [20]. HCT116 cells were maintained and propagated in complete RPMI-1640 medium L-Glutamine (Lonza Verviers SPRL, Belgium, cat#12-604F) which supplemented with 10% fetal bovine serum (FBS) (Seralab, UK, cat# EU-000-H), and 1% antibiotic (Antibiotic antimycotic, Biowest, cat# L0010). The cells were reared in humidified incubator with 5% CO_2 at 37 °C, sub-cultured using 0.25% (w/v) trypsin/EDTA, and then counted using trypan blue staining and investigated under inverted microscope according to the standard tissue culture technique [21, 22].

Evaluation of cytotoxicity by MTT assay

The cytotoxic effect of the NTZ (purchased from COPDA Pharma, Egypt, cat#7080316154) on colorectal cancer cell line was evaluated by the cell viability colorimetric assay according to our previous publications using MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) dye [21, 22]. 5-FU is a standard reference drug was used as a positive control. Moreover, we tested the synergistic cytotoxic effect of both NTZ and 5-FU at the same indicated concentrations. Briefly, HCT116 (1×10^4 cells/well) were seeded in a 96-well plate in triplicate and were allowed to adhere for 24 h. NTZ were dissolved in 500 µl Dimethyl sulfoxide (DMSO) to have stock solution of 100 mM, then various concentrations of NTZ or 5-FU were prepared by diluting in complete medium to have five final concentration of 0.01, 0.1, 1, 10, and 100 µM. Worth noting, the final concentration of DMSO in the culture medium didn't exceed 0.2% (v/v) [2]. Treated and non-treated cells were allowed to grow for 48 h. 10 µl of MTT (5 mg/ml in PBS w/o Ca, Mg, Lonza Verviers SPRL Belgium, cat#17-516F) was added in each well four hours before completion of incubation. After the incubation with MTT, color was developed by adding 100 µl of DMSO to each well, and measured at 490 nm using Bio-Tek microplate reader. The experiment was conducted six times. Data were calculated as percent of cell viability by the following formula: % cell viability = (Mean absorbance in test wells / Mean absorbance in control wells) × 100 [3], then IC_{50} was calculated.

Moreover, the morphology of the HCT116 cells treated with NTZ or 5-FU were examined using inverted microscope and captured by SONY CYBER-SHORT [17].

Flow Cytometer analysis

Cell cycle analysis and apoptotic assay were carried out in details according to these publications [23, 24]. HCT116 cells were seeded (1.0×10^6 cells/ flask) for 24 h, then cells were treated with the IC_{50} value (11.20 µM) of NTZ. After 48 h incubation, the cells were trypsinized, harvested, and fixed following the instructions mentioned in Annexin V-FITC Detection Kit (Catalog #: K101-25, BioVision), and propidium iodide stain. Then flow cytometry analysis was followed up, determining in which phase cells would be arrested and also calculating the percentage of apoptotic cells.

DNA fragmentation assay

DNA fragmentation caused by treatment of HCT116 cells with the IC_{50} of NTZ was detected using Diphenylamine assay. Protocol included the release of nuclear DNA after cell lysis, followed by centrifugation step with generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA, hydrolysis and colorimetrical quantitation diphenylamine (DPA) were performed according to the literature [25].

Gene expression analysis

HCT116 cells were treated with 11.20 µM of NTZ for 48 h, then total RNA was extracted from both treated and non-treated cells using Qiagen RNA extraction. The purity of RNA was recorded using nanodrop spectrophotometer. cDNA synthesis was done, then subsequent qPCR test was performed in a single tube. The perfect primer pairs (Table 1) were selected for the tested genes (BAX, P53, Casp-3, -8, -9, BCL2, IL-6, JAK2, and STAT3) and β-actin as a ELIhousekeeping gene. The results obtained were expressed in cycle threshold (Ct), and relative quantitation of each tested gene was assessed according to the calculation of delta-delta Ct [26, 27].

Western blotting

Protein extraction was done for HCT116 cells treated with the IC_{50} of NTZ for 48 h by ReadyPrepTM kit (Bio-Rad Inc, Catalog #163-2086) according to the manufacturer's instructions, and according to our previous publication [27]. Primary antibodies of Bax (Catalog# sc-7480), Bcl-2 (Catalog# sc-7382), NFκB p50 (Catalog# sc-8414), p53 (Catalog# sc-55476), Stat3 (Catalog# sc-8019), and B-actin (Catalog# sc-8432) were purchased from Santaz Cruz Biotechnology, INC company. Primary antibodies were diluted in TBST according to manufactured instructions. Incubation was done overnight with each primary antibody solution at 4 °C. The

Table 1 Forward and reverse primers used in gene expression analysis

Gene	Forward	Reverse
BAX	5′-GTTTCATCCAGGATCGAGCAG-3′	5′-CATCTTCTTCCAGATGGTGA-3′
P53	5′-CCCCTCCTGGCCCCTGTCATCTTC-3′	5′-GCAGCGCCTCACAACCTCCGTCAT-3′
Casp-3	5′-TGGCCCTGAAATACGAAGTC-3′	5′-GGCAGTAGTCGACTCTGAAG-3′
Casp-8	5′-AATGTTGGAGGAAAGCAAT-3′	5′-CATAGTCGTTGATTATCTTCAGC-3′
Casp-9	5′-CGAACTAACAGGCAAGCAGC-3′	5′-ACCTCACCAAATCCTCCAGAAC-3′
BCL2	5′-CCTGTGGATGACTGAGTACC-3′	5′-GAGACAGCCAGGAGAAATCA-3′
IL6	5′-CCAGCTATGAACTCCTTCTC-3′	5′-GCTTGTTCCTCACATCTCTC-3′
JAK2	5′-AGCAGCAAGTATGATGAGCAAG-3	5′-GAGAAAGGCATTAGAAAGCCTGTAG-3′
STAT3	5′-CTAGACAATATCATCATCGACCTTG-3′	5′-TCCCGCTCCTTGCTGATGA-3′
FASL	5′-CTGAATTCTGACTCACCAGCTGCCATGC-3′	5′-ACTCGAGCTATTAGAGCTTATATAAGCC-3′
β-actin	5′-GTGACATCCACACCCAGAGG-3′	5′-ACAGGATGTCAAAACTGCCC-3′

blot was then washed for 5 min with TBST for 3 times. Incubation for 1 h was done in the HRP-conjugated secondary antibody (Goat anti-rabbit IgG- HRP-1 mg Goat mab -Novus Biologicals) at room temperature. The blot was rinsed 3–5 times for 5 min with TBST, then the immune precipitation was detected using the chemiluminescent substrate (ClarityTM Western ECL substrate Bio-Rad Catalog #170-5060). The chemiluminescent signals were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins against control by protein normalization on the ChemiDoc MP imager.

ELISA assay

ELISA assay for target proteins was done on HCT116 cells treated with the IC_{50} of NTZ. After 48 h, treated and non-treated cells were lysed using cell extraction buffer, the lysate was diluted using standard diluent buffer over the range of the assay and measured for human active caspase-3 content (The Max Discovery™ Caspase-3 Colorimetric Detection Kit Manual (Catalog. no # 5811)), human cytochrome c (Quantikine® ELISA, Human Cytochrome c Immunoassay (cat. no #DCTC0)), and Vascular Endothelial Grwoth Factor (VEGF) (RayBio® Human VEGF-A ELISA Kit (Catalog. no #ELH-VEGF)). All procedure for standard ELISA techniques were done according to our previous publication [27].

Statistical analysis

Data were represented by the value of the mean for three different replicates, with standard error of mean (SEM). All statistical analysis was done as using GraphPad Prism software version 5.01. Statistical difference between two groups was examined using Student's *t*- test while one-way ANOVA was used when more than two groups were tested. The significance level was set at $P < 0.05$.

Results

Molecular docking

The in silico study using molecular modeling was used to test the possible mode of action of NTZ compound compared to 5-FU against two different proteins Janus kinase 2 (JAK2), and B-cell lymphoma-2 (BCL2), which are implicated significantly in cancer disease. These two different targets were selected based on their potential roles in the progression of the CRC. Therefore, targeting of these macromolecules represents potential targets for CRC therapy. The cocrystal ligands of the two proteins were redocked to assure the accuracy of the docking parameters and methods used, and the difference of RMSD value between cocrystal ligands to the original cocrystal ligand was less than 2 Å (for JAK2, RMSD = 0.583 Å), and (for BCL2, RMSD = 1.343 Å) indicating the accuracy of the docking protocols and parameters.

The results of the molecular docking of the *NTZ* and *5-FU* against the receptor JAK2 (PDB: 5AEP) showed that NTZ had better binding affinity compared to 5-FU, as NTZ displayed free energy of binding less than 5-FU (− 7.8, and − 5.0 kcal/mole, respectively) (Table 2). Hydrophobic interaction for NTZ which was documented with 5 amino acid residues (LEU932, LEU983, VAL863, VAL911, and PHE863), and hydrogen bond formation with the key amino acid residue (ARG980, with length of 2.973 Å) (Table 3 and Fig. 2). Moreover, NTZ had less free energy of binding less than 5-FU when it was docked against BCL2 protein (− 6.4, and − 6.2 kcal/mole, respectively) (Table 2), and NTZ formed 2 hydrogen bonds with the key amino acid residues in BCL2 pocket (ARG143, with length of 2.351 Å, and ARG143, with length of 2.955 Å) (Table 3 and Fig. 3). Based on these results, NTZ considered to be promising anti-cancer agents against CRC.

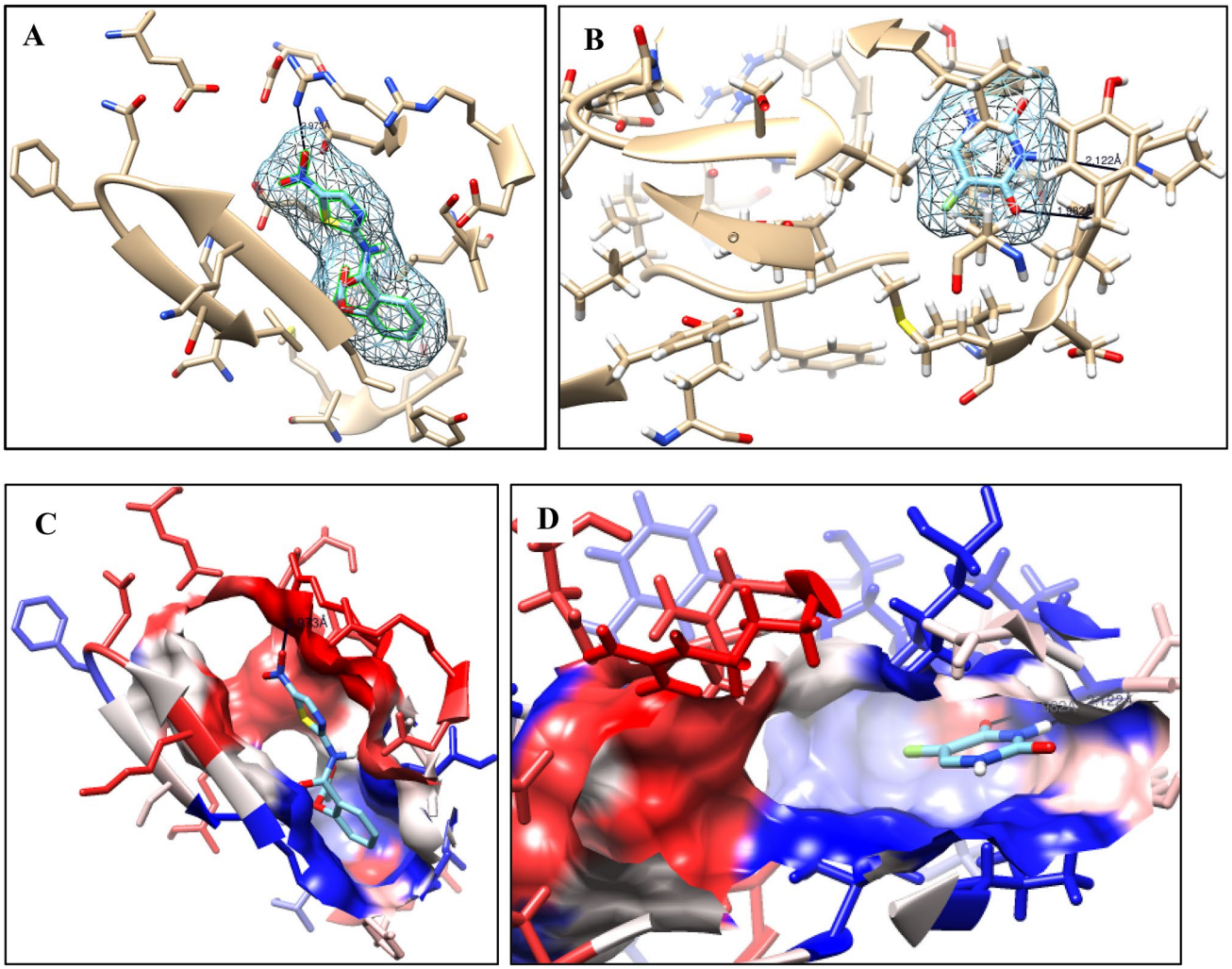

Fig. 2 3D presentation for the interaction of NTZ and 5-FU with JAK2 protein: **a** hydrogen bond interaction in the pocket of JAK2 protein with NTZ, **b** hydrogen bond interaction in the pocket of JAK2 protein with 5-FU, **c** Hydrophobic interaction (indicated by blue color) in the pocket of JAK2 protein with NTZ, **d** Hydrophobic interaction (indicated by blue color) in the pocket of JAK2 protein with 5-FU

Cytotoxic effect of tested compounds on colorectal cancer

The cytotoxicity of NTZ was investigated against CRC cell line (HTC116) at concentrations (0.01, 0.1, 1, 10, and 100 µM) using MTT colorimetric assay. Data illustrated in Fig. 4 showed a concentration-dependent reduction in the percentage of viable colon HCT116 cells after 48 h from treatment with different concentrations of the NTZ alone and in combination with 5-FU in comparison to individual 5-FU. The IC_{50} of NTZ, or 5-FU, or NTZ and 5-FU against HTC116 were 11.20 µM, 23.78 µM, and 26.30 µM; respectively (Table 4). The morphological assessment for the HCT116 treated with the IC_{50} of NTZ, 5-FU and combination of NTZ and 5-FU showed that, treatment with NTZ alone was able to change the morphology of cells dramatically (*Black arrows*), which was depicted by increasing cell lysis, shrinkage of the cells, and apoptotic bodies (Fig. 5). These results depicted that NTZ is more cytotoxic to the CRC more than 5-FU, and even the combination of NTZ and 5-FU did not increase the cytotoxic effect against HCT116. Therefore, we decided to study in details the molecular mode of action of the NTZ on CRC.

Cell cycle and apoptosis analysis

To investigate whether NTZ induces perturbations in the cell cycle, identical HCT116 cell-treated with 11.20 µM for 48 h and compared to non-treated cells. The percent of cells accumulation in each phase of cell progression, and apoptotic study were further checked based on flow cytometry analysis. Treatment of HCT116 cells with NTZ resulted

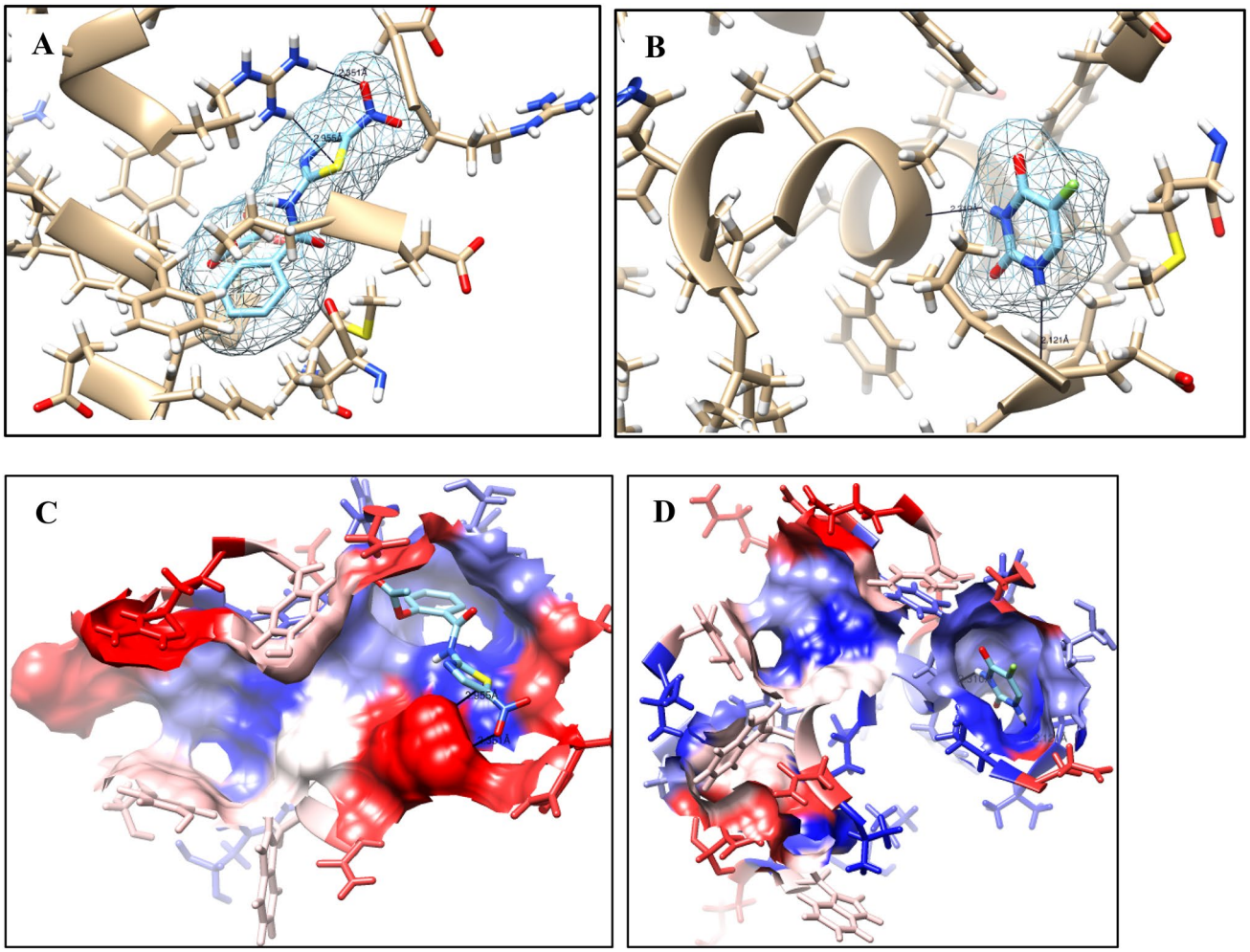

Fig. 3 3D presentation for the interaction of NTZ and 5-FU with BCL2 protein: **a** hydrogen bond interaction in the pocket of BCL2 protein with NTZ, **b** hydrogen bond interaction in the pocket of BCL2 protein with 5-FU, **c** Hydrophobic interaction (indicated by blue color) in the pocket of BCL2 protein with NTZ, **d** Hydrophobic interaction (indicated by blue color) in the pocket of BCL2 protein with 5-FU

Table 2 The results of molecular docking of NTZ and 5-FU against JAK2 and BCL2

Compounds	Free energy of binding (Kcal/mole)	
	JAK2	BCL2
Reference ligand	− 9.1	− 10.6
5FU	− 5.0	− 6.2
NTZ	− 7.8	− 6.4

in cessation of cell proliferation, which was depicted by decrease number of cells in S phase (27.69%) compared to (41.25%) in control cells, as transition of cells from G1 to S phase is key process in controlling cell proliferation. Moreover, NTZ treatment resulted in cell accumulation in G2/M phase (35.94%) compared to untreated control (11.77%) (Fig. 6a, b, Table 5). Further on, NTZ treatment-induced apoptosis significantly in CRC cells (17.22%) compared to untreated cells (1.75%) (Figs. 6c, d, and 7).

DNA fragmentation assay

To detect the percentage of DNA fragmentation associated with NTZ treatment for HCT116 cells, DPA colorimetric assay was applied [28]. This method was based on the notion that extensively fragmented double-stranded DNA can be separated from chromosomic DNA upon centrifugal sedimentation and colorimetrical quantitation by staining with diphenylamine (DPA), which binds to deoxyribose. Figure 8 outlined the percentage of fragmentation in HCT116 cells treated with the IC50 of NTZ and control untreated cells. This data showed high significant ($P < 0.001$) DNA fragmentation caused by NTZ (38.8%) relative to control

Table 3 The results of Hydrogen bond interaction of NTZ and 5-FU against JAK2 and BCL2

Compound	Receptor	No. of H-bonds	Length of H-bonds	Formed amino acids with H-bonds
Reference ligand	JAK2	2	3.631 Å	ARG980
			3.142 Å	LEU932
5FU	JAK2	2	1.982 Å	LEU932
			2.122 Å	LEU932
NTZ	JAK2	1	2.973 Å	ARG980
Reference ligand	BCL2	–	–	–
5FU	BCL2	2	2.310	ALA146
			2.121	VAL130
NTZ	BCL2	2	2.351 Å	ARG143
			2.955 Å	ARG143

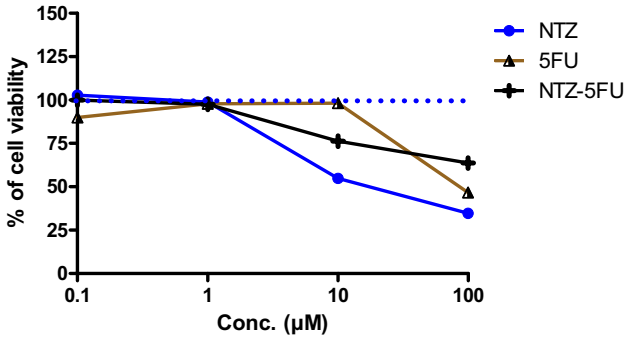

Fig. 4 Cytotoxic effect of NTZ, 5-FU, and NTZ + 5-FU against HTC116 cells. 1×10^4 cell/well of HCT116 cells were treated with serial dilution of the NTZ or 5-FU or combination of NTZ + 5-FU for 48 h cytotoxic effect was detected by MTT assay. The figure shows the percentage of cell growth viability compared to control which were untreated cell. Error bars were values of SEM for six different replicates, ($n = 6$)

Table 4 IC_{50} values for NTZ, 5-FU, and NTZ + 5-FU against colon (HTC116) cell line

Sample	IC_{50} (μM)
NTZ	11.20
5FU	23.78
NTZ + 5FU	26.30

sample, thus this data confirmed high cytotoxicity of NTZ on colorectal carcinoma cell line.

Effect of NTZ on apoptosis-related signaling pathway

Gene expression analysis for key genes modulating CRC progression was done. HCT116 cells were treated with the IC_{50} value of the NTZ for 48 h at 37 °C, and then the treated cells plus control were collected for real-time PCR analysis for the following 10 targets (BAX, Casp3, casp8, casp9, FASL, BCL2, IL-6, JAK2, and STAT3) using β-actin as housekeeping gene. Results in Fig. 9 demonstrated that NTZ strongly induced the expression BAX (fold change (FC) = 4.06), P53 (FC = 3.25), Casp-3 (FC = 4.21), Casp-8 (FC = 2.09), Casp-9 (FC = 6.30), and FASL (FC = 2.77), while downregulation of BCL2 (FC = 0.48), IL6 (FC = 0.30), JAK2 (FC = 0.78), and STAT3 (FC = 0.54) was observed. Further on, and to validate the effect of NTZ on CRC on protein level, western blot was done. Protein expression of Bax, P53, NF-kb, Bcl2, Stat3, and B-actin was depicted, and band intensity was quantified using ChemiDoc MP imager. Results in Fig. 10 showed high expression of both P53, and Bax proteins in NTZ treated cells compared to untreated control, while downregulation of Bcl2, Stat3, NF-kb in NTZ-treated cells compared to untreated control. These results are in a good agreement with the gene expression data.

Effect of NTZ on Casp3, Cyto-c, and VEGF levels detected by ELISA

Further on, to confirm the induction of apoptosis by via mitochondrial pathway, HCT116 cells were treated with the IC_{50} of NTZ, and the activity of Casp3, Cyto-c, and VEGF were determined using ELISA assay. Results in Fig. 11 showed significant ($P < 0.05$) induction of both caspase-3, and cytochrome c after treatment of HCT116 with NTZ, while significant ($P < 0.05$) repression of VEGF was associated with NTZ treatment compared to untreated HCT116 cells.

Discussion

Numerous signaling and molecular cellular cascades are involved in carcinogenesis, progression, and prognosis of CRC. The genetic and epigenetic pathways with a significant

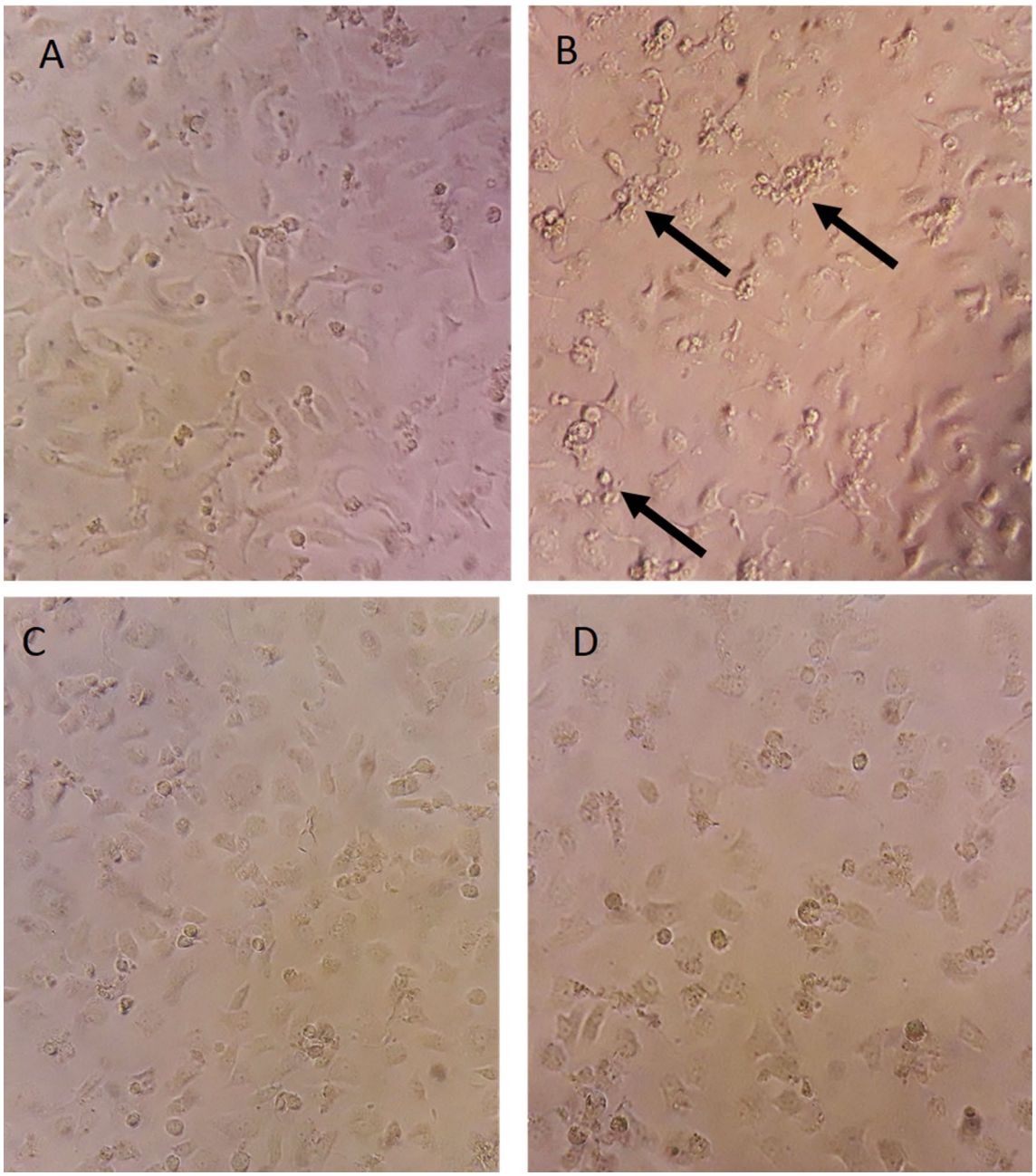

Fig. 5 The morphology of HCT116 cells upon treatment with the IC_{50} values after 48 h. **a** Control, **b** NTZ, **c** 5-FU, and **d** NTZ and 5-FU

role in these processes include chromosomal instability (CIS), microsatellite instability (MSI) and DNA methylation. Alongside these machineries, inflammation and dysregulated apoptosis are key pathways; not only for initiation but also for establishment, proliferation, and cell cycle advancement of CRC cells [29–31]. The current CRC therapeutic challenges are targeting these pathways. However, targeting should ideally manipulate all activated CRC molecular regulators to reduce the occurrence of chemo-resistance and relapse [32–34].

NTZ, a thiazole-containing derivative [35], is one of the promising anticancer drugs. In several pre-clinical studies, NTZ exhibited antiproliferative effects in ovarian cancers, glioblastoma, and CRC via modulating several molecular pathways as UPR, the oncogene c-Myc, detoxification of chemotherapies, immune/inflammatory/apoptotic and autophagic responses [5–7, 14, 36, 37]. In human CRC cell lines, NTZ affected the AMPK, c-Myc, mTOR, and Wnt downstream signaling pathways [7]. Evidently, thiazole derivatives inhibit mitochondrial respiration, prompt

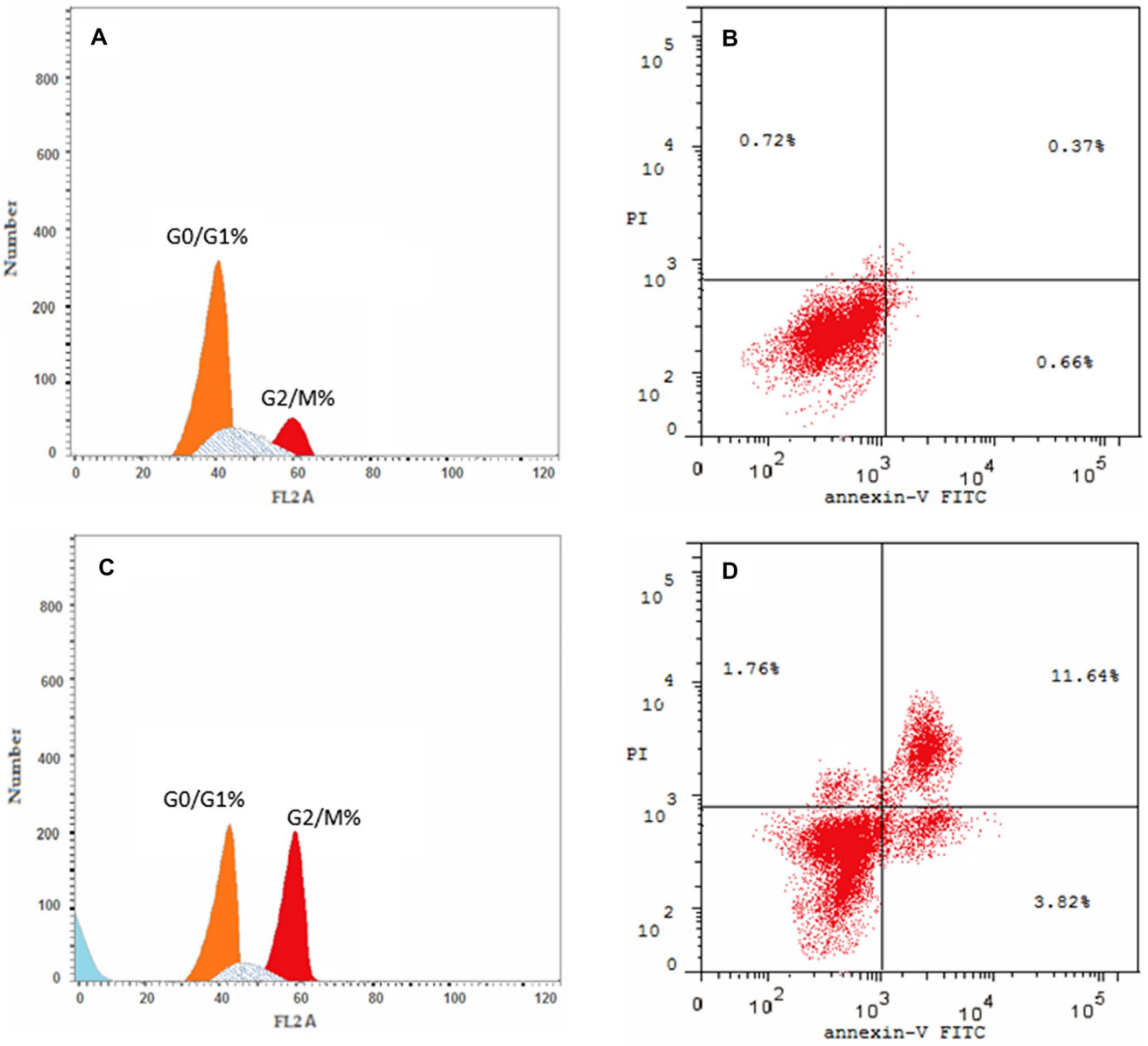

Fig. 6 DNA histograms of cell cycle analysis of HCT116 cells (**a**, **b**) and apoptosis analysis (**c, d**). The percentage and distribution of cells in pre-phase, G1, S and G2/M phase of the cell cycle were indicated (**a** untreated control cells, **b** HCT116 cells after treatment with IC_{50} of NTZ (11.20 µM) for 48 h). The percentage of cell stained with PI/Annexin were indicated (**c** untreated control cells, **d** HCT116 cells after treatment with IC_{50} of NTZ (11.20 µM) for 48 h)

Table 5 DNA content in each phase of the cell cycle for HCT116 cells treated with the IC_{50} of NTZ for 48 h, compared to non-treated control

Sample	G0/G1 (%)	S (%)	G2/M (%)
Control	46.94	41.29	11.77
NTZ	36.37	27.69	35.94

mitochondrial depolarization, DNA fragmentation and G1 phase of cell cycle arrest [36]. These previous mechanistic studies elucidated partially how NTZ induces CRC cell death by apoptosis. In the current study, the NTZ's modulatory roles on the p53/Bcl-2/Bax/caspase-9/caspase-3, the p53/FASL/caspase-8, and the IL-6/JAK2/STAT3 were explored on the human CRC cell line (HCT116).

The present study demonstrates NTZ-concentration-dependent cytotoxicity on CRC. NTZ or 5-FU incubations decreased the proliferation and viability of human HCT116 cells while incubation of cells with the combination NTZ

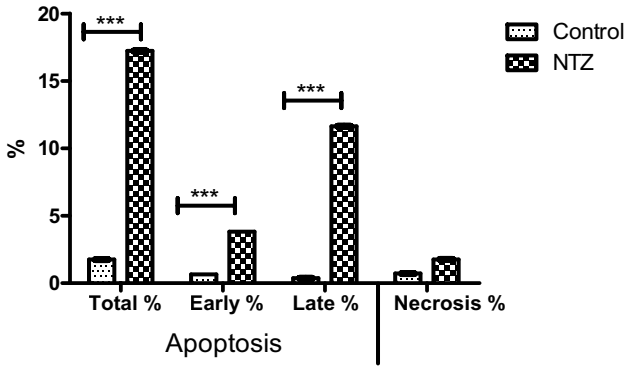

Fig. 7 Schematic diagram outlined apoptotic percentage of HCT116 cells treated with the IC50 of NTZ for 48 h of treatment in different phases, ($n=3$), ***$P<0{,}001$ compared to control

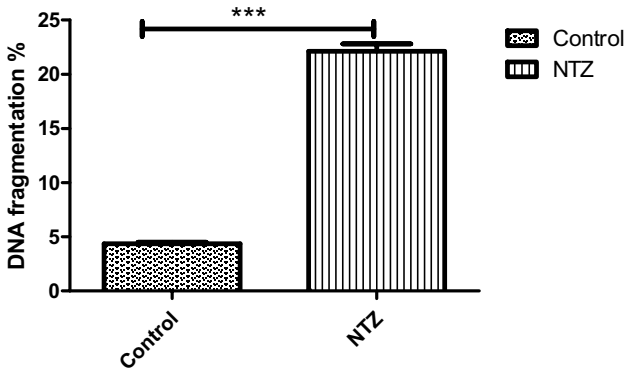

Fig. 8 Schematic diagram outlined DNA fragmentation percent of both NTZ treated HCT116 cells and untreated cells, (n = 3), ***$p<0{,}001$ compared to control

and 5-FU did not displayed synergistic cytotoxicity even at higher concentrations. In a surprising manner, NTZ alone showed more reductions in viable CRC cells in comparison with 5-FU alone, or the combination of NTZ and 5-FU. According to these preliminary data, the subsequent investigations in this work were directed toward exploring some possible mechanisms of NTZ cytotoxic effect.

In the current study, NTZ downregulated the genetic expression of IL-6, JAK2, and STAT3 in HCT116 cell lines after 48 h. These finding highlights another NTZ therapeutic target in CRC.

Previous studies validated the role of IL-6/JAK2/STAT3 pathway in cancer. Persistent activation of this pathway upregulates, at expressional levels, a wide variety of inflammatory/immune/apoptotic regulatory genes such as Bcl-2 gene family and VEGF which results in augmented tumor cell invasion, angiogenic characteristics, and metastasis [33, 38–41]. In human CRC with poor prognosis, IL-6 expressions at the genetic and protein levels are upregulated and correlated with tumor cell proliferation and differentiation, angiogenesis, invasion, and migration [5, 14, 42, 43]. The pleiotropic trans-signals of IL-6 activate the IL-6/JAK2/Stat3 downstream cascade in CRC [43, 44]. JAK2 and Stat3 are important biological regulators in a wide variety of normal and abnormal cells as tumor cells. JAK2 controls cellular growth, differentiation, and survival. In a continuation, STAT3 regulates the apoptotic cellular behaviors, angiogenesis, and migration [33, 40, 42, 45].

The inhibition and downregulation of JAK2/STAT3 cascade signaling have distinctive roles in the mitochondrial caspase-dependent apoptotic execution, which were accompanied by modulating of the Bcl-2 gene family, and

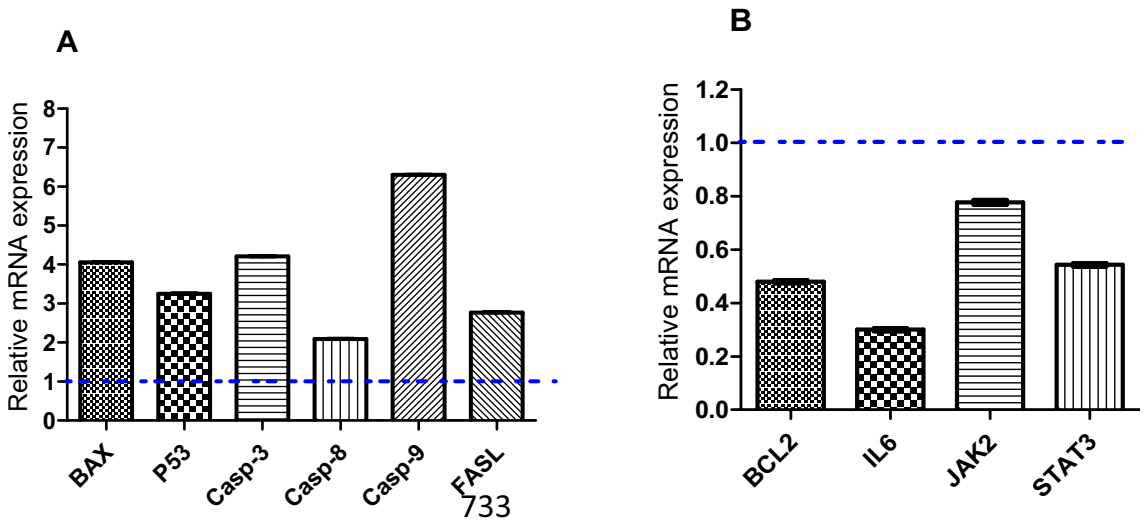

Fig. 9 Relative gene expression analysis of BAX, P53, Casp3, casp8, casp9, FASL (**a**), and BCL2, IL-6, JAK2, and STAT3 (**b**) for HCT116 treated with IC_{50} of NTZ, versus untreated control using real time PCR, while the dashed line represents the control (Fold change = 1) ($n=3$)

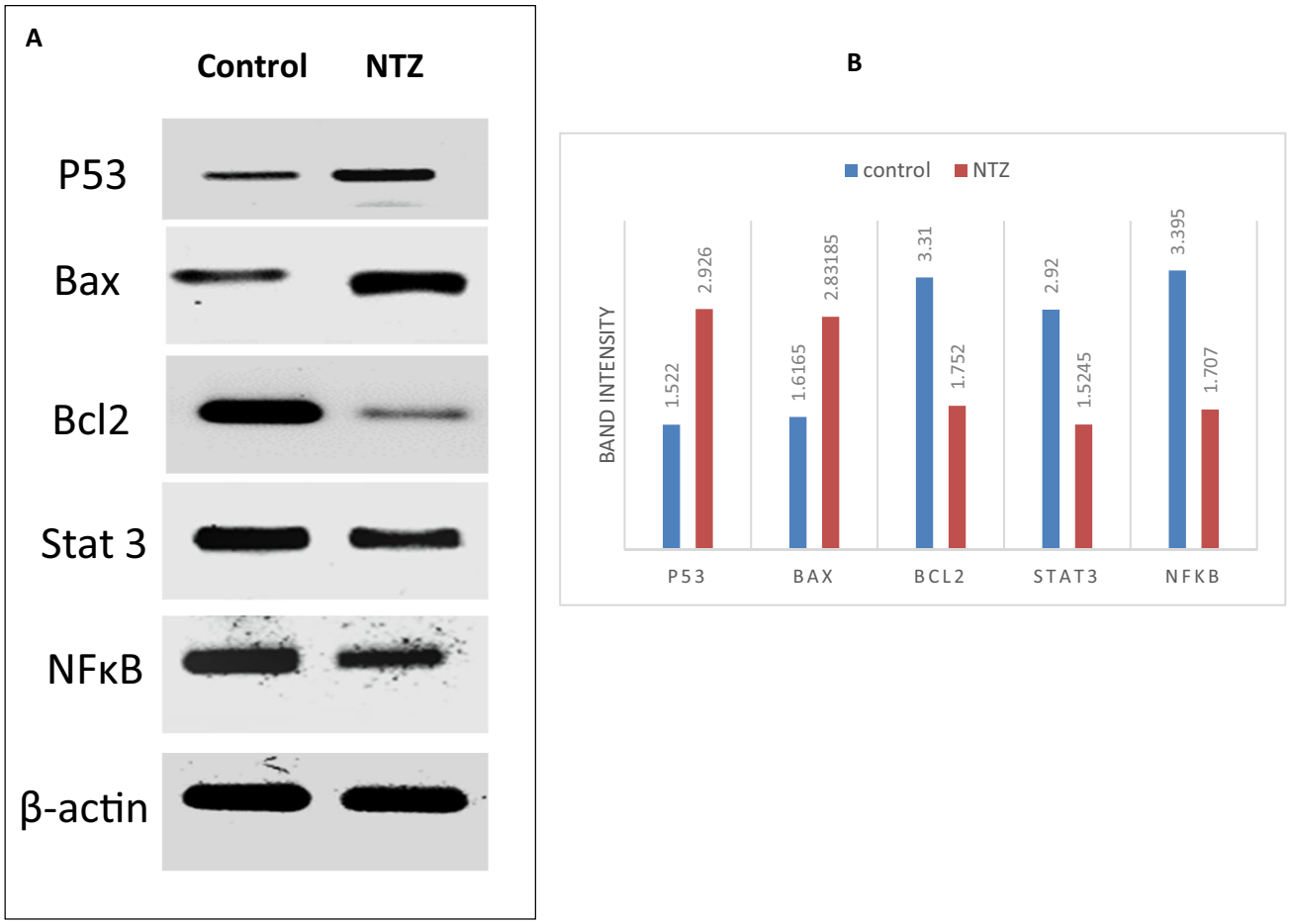

Fig. 10 Protein quantification for HCT116 cells treated with IC_{50} of NTZ, versus untreated control using western blot analysis. **a** Target proteins bands for P53, Bax, Bcl2, Stat3, NF-kb, and B-actin. **b** Band intensity quantification using ChemiDoc MP imager

cytochrome c translocation, and caspases 9 and 3 activation [46, 47]. These evidences support our finding regarding the NTZ-apoptotic enhancement, high levels of cytochrome c, and caspases activation in HCT116 cell lines. In light of previous studies and our findings, targeting the IL-6/JAK2/STAT3 signaling downstream by drugs including NTZ may represent a promising therapeutic strategy for CRC [42].

Apoptosis, a programmed cell death, could be caspase-dependent, and/or non-caspase-dependent. In the extrinsic cascade of apoptosis, several extrinsic activators manipulate p53. The activated p53 attaches to specific DNA sequences activating transcription of several apoptotic genes as Bcl-2 family proteins. The pro-apoptotic protein Bax ameliorates the anti-apoptotic protein Bcl-2 with consequent mitochondrial cytochrome c release in the cytosol of human CRC cells. That release is also controlled by other intrinsic activators of the Bcl-2 family proteins; intrinsic apoptotic cascade. The formed apoptosome complex (cytochrome c, caspase 9, apoptotic protease activating factor-1) activate caspase 9 which then activate the executive caspase 3 [29, 32]. Of notice, in this work NTZ IC_{50} exhibited increase in the percentage of apoptotic cells after 48 h incubation. This was accompanied by enhanced DNA fragmentation of HCT116 cells. Moreover, after NTZ challenging cells demonstrated augmented expression of p53, BAX, and caspases 9 and 3 genes and a reduced genetic expression of Bcl-2. These finding were confirmed by amplified protein expressional levels of cytochrome c and enhanced activity of caspase 3. These results are in line with previous evidences presented by Senkowski and colleagues [7].

Fas (CD95, APO1), one of the tumor necrosis factor receptor superfamily, occurs as a membrane receptor on all types of mammalian cells. In contrary, the physiological ligand of Fas (FasL, CD95L) is selectively expressed on paucity of immune cells and tumor cells. The extrinsic pathway of apoptosis is dependent on the death receptors and its ligands called FasR/FasL. The cytotoxicity mediated by FasL is an important mechanism by which immune cells as cytotoxic T lymphocytes and natural killer cells kill the cancer cells. FasL could induce apoptosis through activation

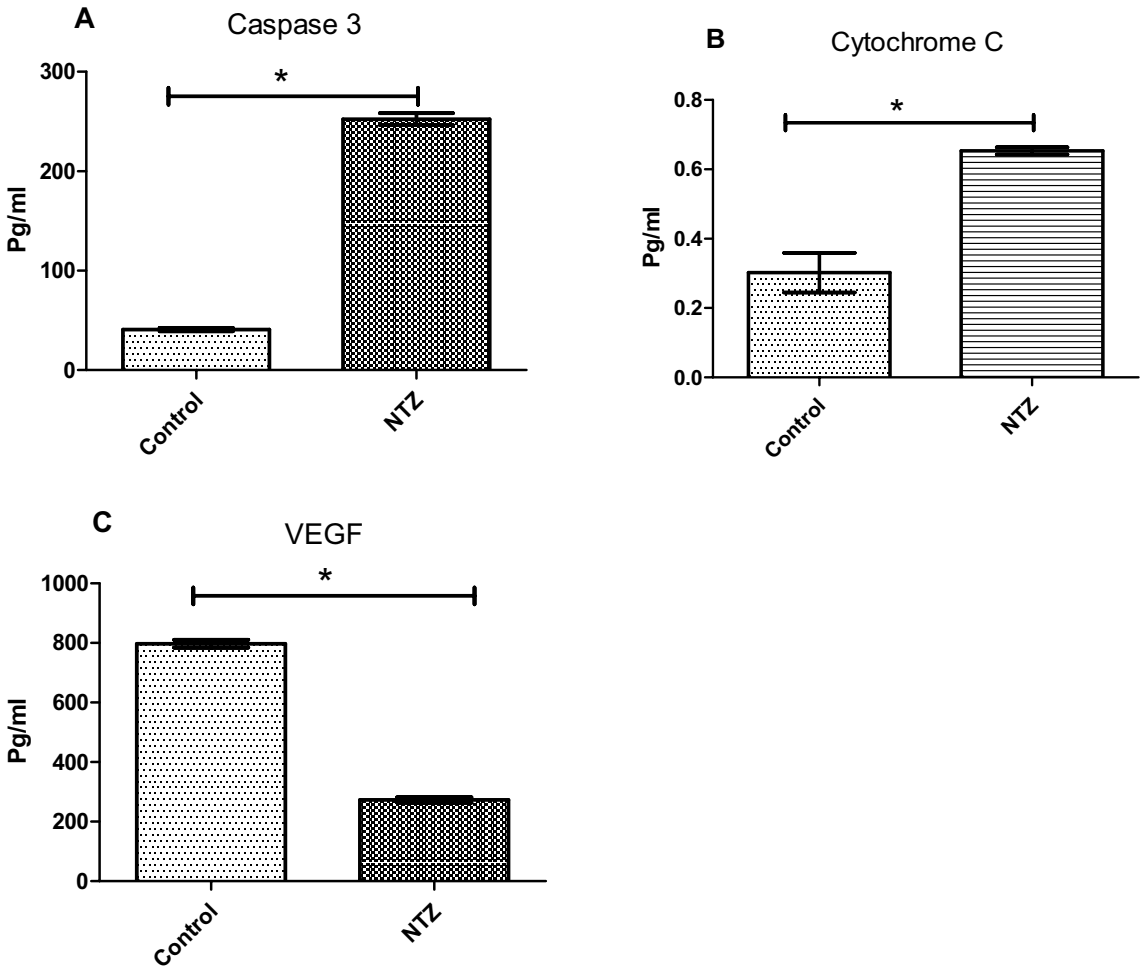

Fig. 11 Schematic diagram illustrating the expression level of Caspase-3 (**a**), Cytochrome C (**b**), and VEGF (**c**) in HCT116 cells treated with the IC_{50} of NTZ after 48 h using colorimetric technique ($n=3$), $^{*}P<0.05$ compared to control

of caspase-8; the extrinsic apoptotic pathway; under the transactivation by active p53. Previous evidences collected from human high-grade CRC displayed dysregulated FasR/FasL signaling cascade. Marked decrease in FasL levels was documented in survived cancer cells' masses with negative prognosis [32, 48, 49]. In the current work, HCT116 cells showed higher genetic expressions of FasL and caspase-8. That was documented in the previously published verifications of the origin of those cells as an aggressive high-grade and poorly differentiated ones. In this study, NTZ application on those cells ameliorated the higher genetic expression of FasL and caspase-8. Moreover, the amplified genetic expression of p53 in the currently used HCT116 cell line documented the p53 wild behavior of the used HCT116 cells. Those recent evidences support a better cytotoxicity of NTZ than 5-FU which has no effect on P53-mediated cell death [50].

After damaging of DNA, p53 plays a crucial role in keeping cells at a checkpoint in a quiescent manner [29, 51]. This braking action provide cells with the time needed for reversal and regeneration of the damaged DNA. Through the present study, NTZ induced cell cycle arrest in G2/M phase. The NTZ-induced genetic upregulation of p53 in HCT116 cells could be partially a cause for NTZ cell cycle arrest actions.

Former scientific knowledge evidenced that VEGF has autocrine effects in CRC cells. It is now considered as a prognostic predictor for human CRC behaviors [52, 53]. Targeting the VEGF-regulated angiogenesis provided a fundamental developing anti-cancer modality [53, 54]. The current work demonstrates the reduction of VEGF levels in HCT116 cell lines after challenging with NTZ. This could be as a continuation of IL-6/JAK2/STAT3 pathway downregulation by NTZ, and by its trans-activation of p53. Takahashi and colleagues provided a strong evidences linking the existence of p53 wild type human CRC cells to the activity of VEGF [55].

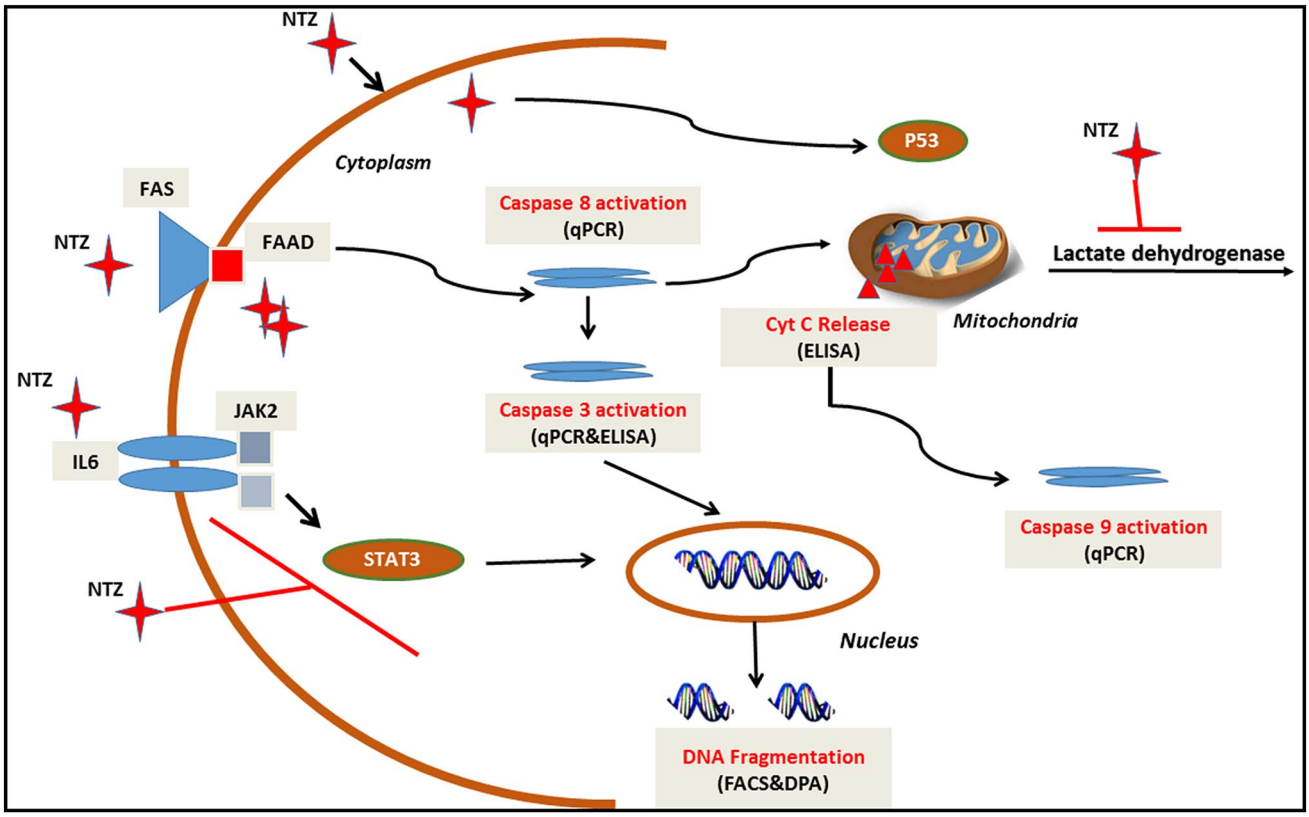

Fig. 12 Proposed model shows the mechanism of action of NTZ in HCT116 cell line

Conclusion

In conclusion, the present work presents another building block in the explanation of the anti-proliferative, apoptotic, and anti-angiogenic actions of NTZ in human CRC HCT116 cell lines (Fig. 12). The IL-6/JAK/STAT signaling, and p53-controlled apoptosis and cell cycle regulation could be considered as anti-cancer targets for NTZ. However, in vivo, and pre-clinical works are needed for more in depth exploration of those signaling and molecular pathways and its cellular and paracrine percussions. Furthermore, the combined regimens of NTZ with other FDA approved cytotoxic therapies for CRC would represent another research area in future studies.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests. Moreover, the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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