Journal of Biochemical and Molecular Toxicology

WILEY

RESEARCH ARTICLE OPEN ACCESS

Promotion of Autophagy and Apoptosis in Colorectal Cancer Exposed to Imatinib and Thymoquinone

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Received: 13 November 2024 | **Revised:** 18 February 2025 | **Accepted:** 13 March 2025

Keywords: AMPK | apoptosis | autophagy | imatinib | Par-4 | thymoquinone

ABSTRACT

Cancer cells possess high proliferative ability and usually override apoptosis and metastasize to distant lesions. Autophagy in cancer cells is a double-edged weapon where a cross-regulation postulation between apoptosis and autophagy exists. The aim of the present study was to investigate the effect of adding Thymoquinone (TQ) to Imatinib (IM) in HCT_{116} human colorectal cancer cell line model on various apoptotic and autophagy markers. The combination doses of IM and TQ were selected according to our previous study concerned with cytotoxicity and uptake/efflux genes modulation. In the current study, the combination induced autophagy in HCT_{116} cell line which in turn enhanced apoptosis. Moreover, early apoptosis was evidenced. The induction of both autophagy and apoptosis resulted in programmed cell death. The assessment of AMPK, Par-4, apoptosis markers, colony formation assays, flow cytometry and autophagy detection by acridine orange proved this rapport.

1 | Introduction

Carcinogenesis comprises a multitude of progressive steps that usually begin with mutations and can end with metastasis [1]. Because of these genetic alterations cancer cells encounter unwarranted survival and proliferative capabilities [2]. Colorectal cancer (CRC) is considered a prominent proof of metastasis following the primary carcinoma where almost 20%–30% of the patients suffer metastasis to various organs [3].

Apoptosis is a ubiquitous cellular program adopted by cancer cells during the course of tumorigenesis that aims to eliminate damaged or abnormal cells [4]. However, cancer cells can override apoptosis by amplifying the antiapoptotic genes or downregulating the proapoptotic ones [5].

On the other hand, autophagy is a vacuolar, lysosomal pathway involved in stress for the degradation of misfolded proteins and damaged organelles in eukaryotic cells [6]. Autophagy maintains the intracellular homeostasis in normal cells against neoplastic transformation [7]. Unfortunately, autophagy can sometimes result in cancer cells being more likely to survive than normal cells in hypoxia and starvation, as well as during anticancer therapy [8]. The role of autophagy in cancer cells is dichotomous and a thorough understanding of the cross-regulation between apoptosis and autophagy is highly demanded to reveal the mechanisms of resistance to chemotherapy and identify novel anticancer targets [9]. Genetic disruption can lead to loss of apoptotic control, stimulate angiogenesis and increase invasiveness of cancer cells [10]. It was evidenced that targeting cancer cells by conventional cancer therapies as cytotoxic drugs or radiation can activate both apoptosis and autophagy [11]. Targeted therapy drugs as imatinib (IM) stimulated autophagy to help cells to survive under stress and to mediate the acquired resistance phenotype against chemotherapy [12]. However, excessive autophagy can promote apoptosis under certain conditions [13]. Furthermore, downregulation of autophagy can promote carcinogenesis [14]. The synchronization and balance between autophagy and apoptosis is complex and has

Summary

- The combination of IM and TQ induced autophagy in HCT_{116} cell line which in turn enhanced apoptosis.
- The combination inhibited colonies formation as well as the increments of various apoptotic markers.
- AMPK, when senses the rising autophagy, it will always activate apoptosis in a stress-defense response.
- We can benefit from the addition of TQ to IM to minimize the resistance cancer cells develop against IM.

been associated with both the induction and the inhibition of neoplasia [15].

Thymoquinone (TQ), a natural compound extracted from *Nigella sativa* with chemo-preventive properties [16]. In-vitro tests showed it was an histone deacetylase (HDAC) inhibitor and could kill colon, prostate and pancreatic cancer cells affecting important genes such as p53 [17]. Furthermore, TQ abolished the expression of several inflammatory cytokines including TNF-α, interleuksin-1β, interleukin-8, and Cox-2 [18]. In previous research, we studied its cytotoxic effect on human colorectal cancer cell line HCT_{116} and it proved to chemosensitize IM [19]. The mechanisms underlying its anticancer role include anti-proliferation, apoptosis induction, cell cycle arrest and antiangiogenesis [20, 21]. In addition, TQ was found to exhibit anticancer activity through the activation of caspases and generation of ROS [22]. Furthermore, TQ blocked angiogenesis in vitro and in vivo with rare chemotoxic side effects [23]. It also proved to downregulate the expression of telomerase enzyme in thyroid cancer cell lines [24]. There is a growing need for innovative antineoplastic drugs especially with the increasing concern over the efficacy and safety of many conventional therapies [22]. The scope of resistance to IM cannot be considered narrow, and furthermore, the clinical response in several patients is hardly sustained [25]. This study aimed to investigate the synergistic effects of TQ when combined with IM in HCT_{116} cell line. We chose TQ for several reasons including being of natural origin given that herbal medicine possesses potent bioactive properties and relatively low toxicity, minimal resistance, contributing to improved quality of life during treatment, in comparison to chemotherapeutic agents as IM [26]. In addition, the antioxidant properties of TQ have garnered significant attention as they demonstrated a protective role against cancer progression [27]. As one of the most challenging tasks concerning cancer is to induce apoptosis in malignant cells, our research aimed to reveal the potential of TQ to overcome chemoresistance caused by IM by studying autophagy and its influence on apoptosis, being an important defense mechanism for cancer tissue survival.

2 | Materials and Methods

2.1 | Drugs and Chemicals

Thymoquinone (TQ) and Imatinib base (IM); were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Zhejiang Wuyi Pharmaceutical Co., (Shanghai, China), respectively. Each drug was freshly dissolved in dimethyl sulfoxide (DMSO) and the stock solutions (10 mM) obtained were stored at −20°C. Before each experiment, the drugs were serially diluted in RPMI-1640 medium immediately. The final concentration of DMSO should never exceed 0.1% (v/v) in all samples. All other chemicals were of analytical grade and of highest purity commercially available.

2.2 | Human Cancer Cell Lines and Cell Culture

The human colorectal cancer cell line HCT_{116} was obtained from American Type Culture Collection (ATCC) and maintained by serial subculturing at the National Cancer Institute, Cairo University, Cairo, Egypt. HCT_{116} cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. HCT_{116} cells culture was incubated in a humid atmosphere with 5% CO_2 supply at 37°C.

2.3 | Colony Formation Assay

Cells were seeded in appropriate dilutions to form colonies. One-day post-seeding, the cells were treated with control, 25 μM Imatinib, 10uM Thymoquinone and the combination (25 μM IM+10 μM TQ) for 48 h. fixed with glutaraldehyde (6% v/v) and stained with crystal violet.

2.4 | Determination of mRNA Expression of Apoptotic Genes by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

HCT_{116} cells were seeded at a density of 250×10^3 cell/mL in RPMI-1640 media. After 24 h, cells were treated with 25 μM IM, 10 μM TQ or their combination for 24 h, and then cells were harvested. Total RNA was isolated by using Trizol Reagent (Invitrogen, CA, USA). The quality and the quantity of the RNA were determined with absorbance analysis by nanodrop (Thermo Fisher, UK). cDNA was prepared from RNA (2000 ng) in a 20 μL reaction using High-capacity cDNA archive kit (Applied Biosystem, California, USA). Thermal cycling was commenced using thermocycler (Biometra, Germany) according to the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and 4°C for ∞.

Notably, qRT-PCR of GAPDH, BAX, BCL-2, BCL-XL, AMPK, and par-4 were performed in triplicate on the thermocycler StepOne real-time PCR System (Applied Biosystems, CA, USA) using SYBR green master mix (Thermo Fisher Scientific, UK). Fast amplification parameters were as follows: 50°C for 2 min, an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C for 15 min, annealing at 56°C for 1 min, then extension at 72°C. Primer-set sequences are described in Table 1. Quantitative analysis of data was performed by using the $2^{-\Delta\Delta C}t$ according to Livak and Schmittgen method [28]. Values were normalized to GAPDH.

TABLE 1 | Oligonucleotides used in the qPCR analysis.

Gene	Forward Primer	Reverse Primer
GAPDH	GTGGAGTCCACTGGCGTCTT	GCAAATGAGCCCAGCCTTC
BAX	CCT TTT CTA CTT TGC CAG CAAAC	GAG GCC GTC CCA ACCAC
BCL-2	ATGTGTGTGGAGAGCGTCAACC	GCATCCCAGCCTCCGTTATC
BCL-XL	GAGCTGGTGGTTGACTTTCTC′	TCCATCTCCGATTCAGTCCCT
Par-4	CCAGCGCCAGGAAAGGCAAAG-3′	CTACCTTGTCAGCTGCCCAACAAC-3′

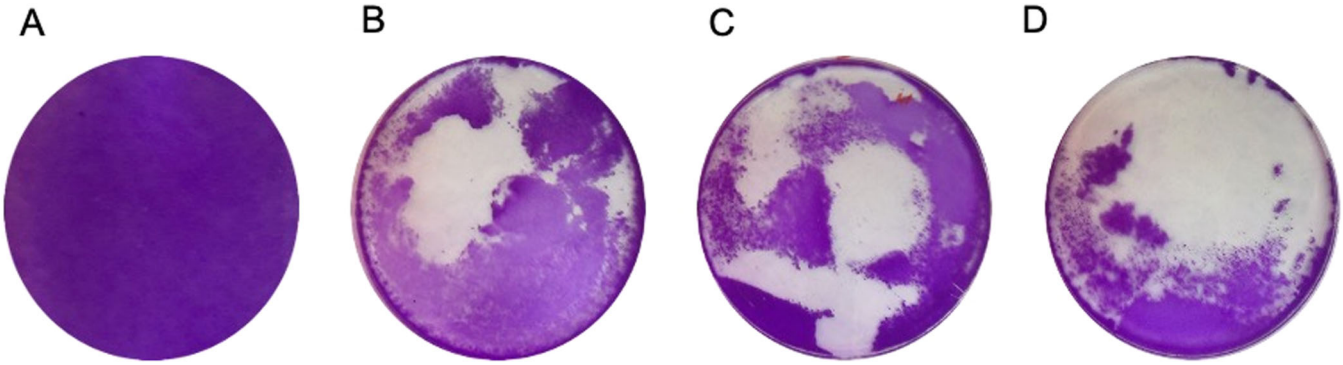

FIGURE 1 | Response of human colorectal cancer cell line HCT_{116} to 25 μM Imatinib mesylate (IM), 10 μM Thymoquinone (TQ) and combination of 25 μM IM + 10 μM TQ on colony formation assays. (A) control, (B) 25 μM IM, (C) 10 μM TQ and (D) combination of 25 μM IM + 10 μM TQ.

2.5 | Analysis of Apoptosis by Flow Cytometry

HCT_{116} cells were plated in 75 mL tissue culture flask with 1×106 cells. After treatment with drugs by 24 h, the cells were harvested and centrifuged at 500*g* at 4°C for 10 min and the cells were stained, and DNA content was quantified by flow cytometry. Annexin V-FITC apoptosis detection kit (Beckman Coulter, California, USA) was used as manufacturer's recommendation by using (Beckman Coulter EPICS XL flow cytometer, California, USA).

2.6 | Autophagy Detection by Acridine Orange (AO)

HCT_{116} cells were seeded at a density of 2×10 cells/well in six well plates. After 24 h, the medium was replaced by drug-free media, 25 μM of IM, 10 μM of TQ and the combination (25 μM IM + 10 μM TQ). After 48 h of treatment, cells were incubated with medium containing AO for 30 min. AO was removed and fluorescent micrographs were taken using an inverted microscope (Nikon, Japan) supplied with blue (excitation BP: 460–490) and green filters (excitation BP: 510–550) and images were acquired using a digital camera (Nikon, Japan).

2.7 | Statistical Analysis

Data are represented as mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) test. To assess the significance of differences, the Tukey *post-hoc* test and unpaired *t*-test were used. A *p*-value of less than 0.05 was considered statistically significant. Graphs and analysis were performed using Prism software program (Graphpad, Prism software, version 5, CA, USA).

3 | Results

3.1 | Colony Formation Assay

Data attained from our previous study showed that TQ synergistically augments the cytotoxic activity of IM in HCT_{116} cells [19]. The IC50 values for IM were significantly reduced to 7.3, 7, and 5.5 μM after combination with (10 μM) TQ and to 5.8, 5.6, and 4.6 μM after combination with (20 μM) TQ for 24, 48, and 72 h. The CI and DRI values indicated a significant synergism in HCT_{116} cells at 24, 48 and 72 h of treatment. In the current study, our previous findings were confirmed by colony formation assays. HCT_{116} cells are significantly more sensitive to combination than control and each drug alone in colony formation assays (Figure 1).

3.2 | mRNA Expression for Apoptotic Genes and AMPK

Further investigation by using qRT-PCR analysis showed that TQ sensitized HCT_{116} cells to IM therapy. TQ induced apoptotic effect of IM via down regulation of the antiapoptotic genes BCL-2 ($p = 0.004$) and BCL-XL ($p = 0.01$) and upregulation of apoptotic gene BAX ($p < 0.001$) and tumor suppressor PAR-4 ($p = 0.002$). Moreover, The BAX/BCL-2 ratio showed significant magnitude in apoptotic effect ($p = 0.001$). Interestingly, IM significantly inhibited AMPK expression while TQ induced AMPK expression as compared to control group. However, the combination neutralized the effect of both drugs (Figure 2).

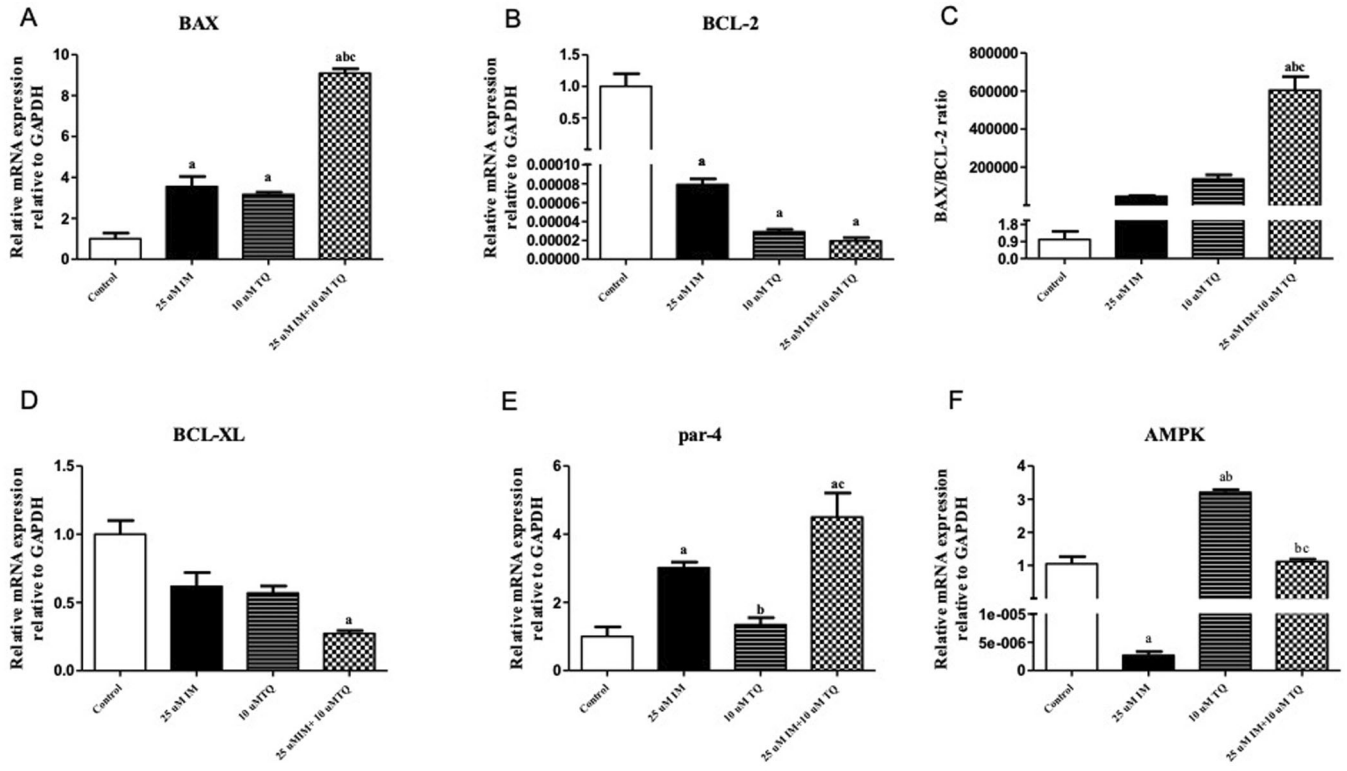

FIGURE 2 | Effect of treatment of 25 μM Imatinib mesylate (IM), 10 uM Thymoquinone (TQ), and combination of 25 μM IM + 10 μM TQ on mRNA expression of Bax, BCL-2, Bax/BCL-2 ratio, BCL-XL, par-4 and AMPK in human colorectal cancer cell line HCT_{116}. Bax expression (A), BCL-2 expression (B), Bax/BCL-2 ratio (C), BCL-XL expression (D), par-4 expression (E), AMPK expression (F). Results are expressed as means ± SD of two independent experiments performed in duplicate. Statistical significance of results was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (a) Significantly different from the control, (b) significantly different from IM, and (c) significantly different from TQ.

3.3 | Flow Cytometry

Further analysis of apoptosis by Flow Cytometry for IM, TQ and their combinations on human colon cancer cells HCT_{116} showed that the total apoptosis rate for control untreated cells, IM, TQ and their combinations were 12%, 23.4%, 22.4%, and 35% respectively. Both IM and TQ produced a significant increase in total and early apoptosis rates as compared to control. It is worth mentioning that the combination showed significant increase in total, early and late apoptosis rates as compared to control and both drugs separately ($p = 0.001$; Figure 3).

3.4 | Autophagy Detection by Acridine Orange

AO was used to detect autophagic vacuoles chromatin condensation. IM induced formation of autophagic vacuoles in HCT_{116} cells. The combination augmented the activation of lysosomes as compared to IM alone (Figure 4).

4 | Discussion

Following therapeutic interventions, cancer cells survive or die via various complex molecular mechanisms including apoptosis and autophagic cell death [29]. Substantial evidence suggests that there is an interactive biology between apoptosis and autophagy in cancer cells [30]. Notably, autophagy; as a mechanism aiding tumor cells to survive bioenergetic stress; is recently viewed as a double-edged weapon whereas the superfluous stimulation of autophagy can also induce apoptosis of the tumor cells [31].

Imatinib, a tyrosine kinase inhibitor (TKI) with known antineoplastic activity, emerged as a target therapy for various malignancies where its target kinases has a pivotal role in cancer cell division and resistance [32]. Previous studies reported the potential effect of IM against chronic myeloid leukemia (CML) with a Philadelphia chromosome and being the first-line anticancer drug for gastrointestinal stromal tumors that metastasize [33]. Unfortunately, approximately 50% of patients develop drug resistance after 2 years of IM treatment [34]. In-depth research has reached different drug-resistance mechanism networks relating IM and other factors [35]. However, its effect on the cross relationship between autophagy and apoptosis is still controversial [36].

On the other hand, TQ exhibits broad spectrum antitumorigenic and anti-inflammatory properties [27]. TQ possesses multitargets against carcinogenesis where it can halt tumor development, invasion, metastasis [37]. It effectively regulates the p53-dependent pathway, promotes apoptosis, and inhibits angiogenesis [38]. It has evidenced to control the expression of Nf-kB and hamper the dissemination of metastatic circulatory malignant cells into the bloodstream [39].

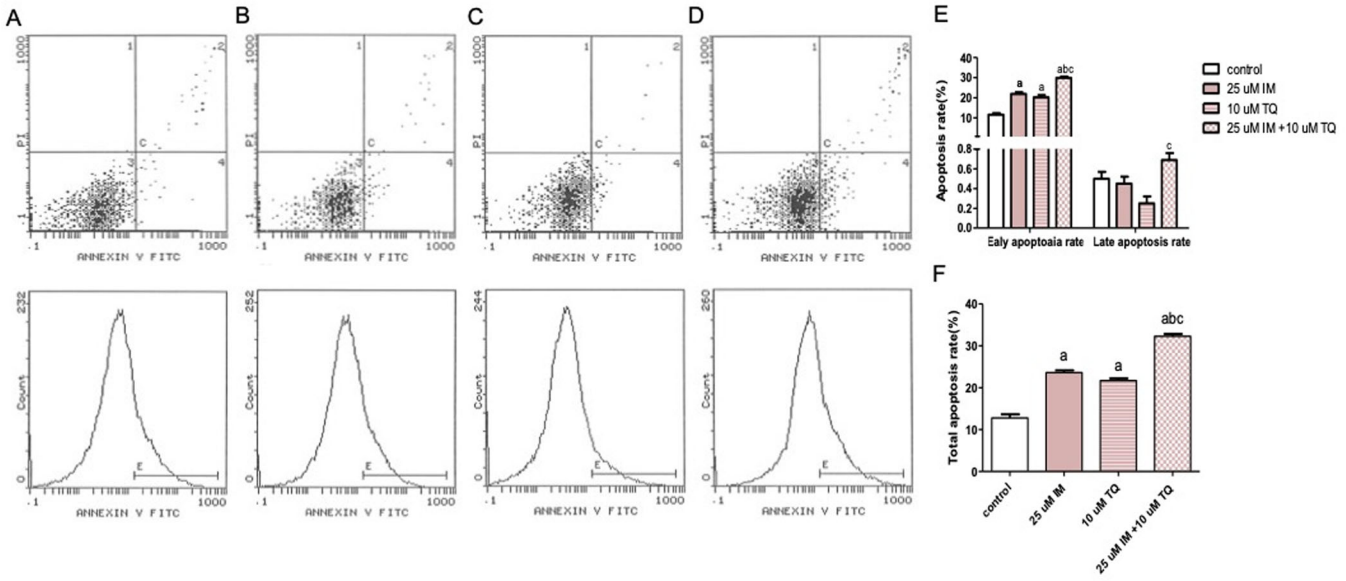

FIGURE 3 | Effect of treatment of 25 μM Imatinib mesylate (IM), 10 uM Thymoquinone (TQ), and combination of 25 μM IM + 10 μM TQ on total apoptotic ratio using flow cytometry in human colorectal cancer cell line HCT_{116}. Flow cytometry scatterplots for (A) control, (B) 25 μM IM, (C) 10 μM TQ, (D) 25 μM IM + 10 μM TQ, (E) quantitative analysis of the early and late apoptotic rate and (F) quantitative analysis of the total apoptotic rate. Results are expressed as means ± SD of two independent experiments performed in duplicate. Statistical significance of results was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (a) Significantly different from the control, (b) significantly different from IM, and (c) significantly different from TQ.

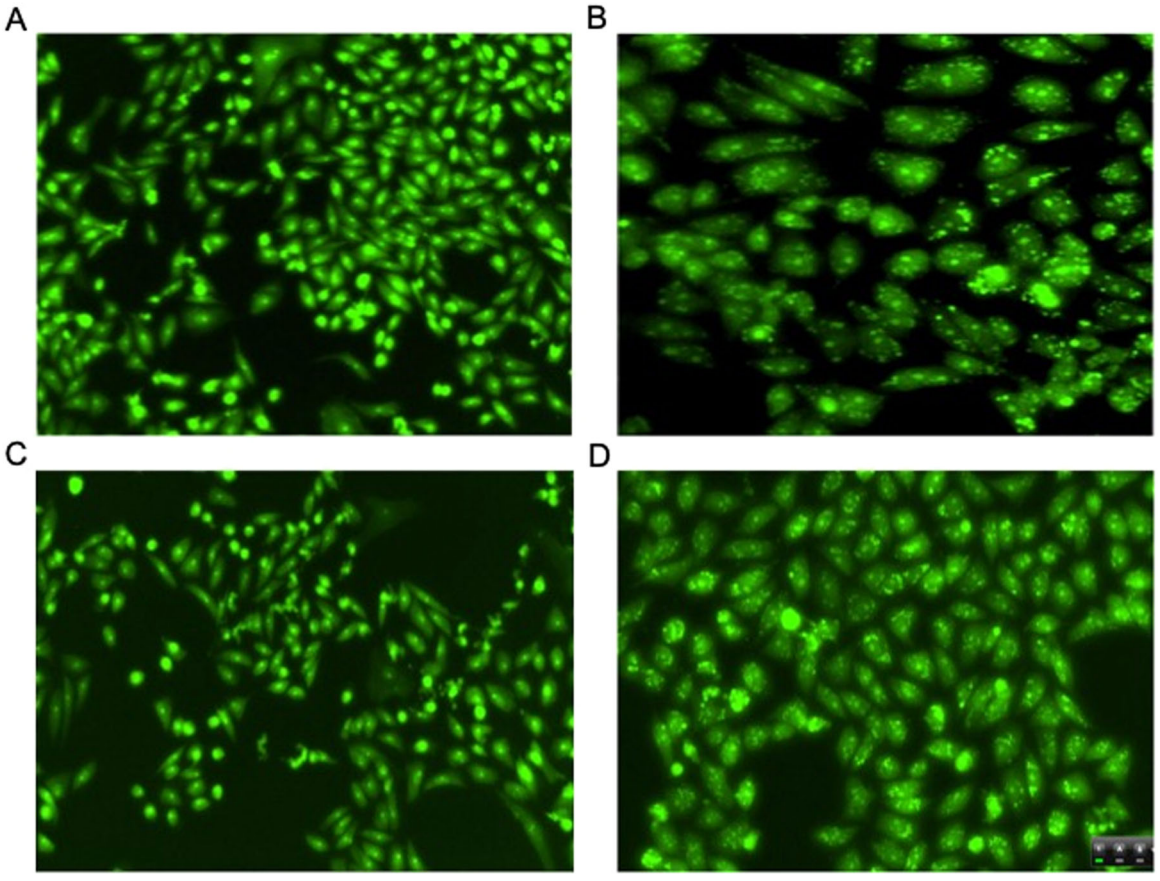

FIGURE 4 | Autophagy detection by acridine orange of human colorectal cancer cell line HCT_{116}, after treatment for 48 h with free drug medium as a control (A), 25 μM Imatinib mesylate (IM), (B) 10 μM Thymoquinone (TQ) (C), and combination of 25 μM IM + 10 μM TQ (D).

Although clinical responses achieved by IM make it the first-line agent for various cancers whatever the phase of the disease, unsustained clinical response emerge in several patients [34]. The acquired resistance to chemotherapy is theorized as the major hindrance to the success of cancer treatment [35]. In turn, the thorough investigation of the underlying mechanisms by which tumors become resistant to a particular agent is key to identifying novel drugs or combination regimens [40]. This need directed our work for examining the possible correlation between autophagy and apoptosis in human colorectal cancer cell line HCT_{116}.

As mitochondria standstill as the pillar for cellular homeostasis, cancer cells reside on it to provide the high need for energy and nutrients to support their rapid growth [41]. In need of a metabolic sensor, AMP-activated protein kinase (AMPK) is considered the main regulator of mitochondrial content where it acts as the main sensor for energy homeostasis in the cell [42]. The activation of AMPK/mTOR pathway was interrelated with CRC proliferation [43]. AMPK enhances autophagosome maturation and fusion with lysosomes [41]. In the formed autolysosomes the hydrolases operate in the acidic medium to destroy the damaged organelles [44].

A proven fact indicates that AMPK downstream signaling increases cell survival during stress times through inducing autophagy [45]. We hypothesized that after exposure of HCT_{116} colorectal cancer cells to drug-induced stress, the cells will commence autophagy to face the stress. To investigate our hypothesis, we focused on assessment of various apoptotic markers to elucidate the mechanism correlating apoptosis and autophagy.

As previously mentioned, cancer is a complex process influenced by a plethora of genetic causes [46]. Autophagy representing one of the important gene transcriptions governs tumorigenesis in multiple ways [47]. In fact, autophagy involves the sequestration of damaged cell structures in double-membrane organelles called autophagosomes [48]. Those autophagosomes fuse with lysosomes in which internal content is degraded by acidic lysosomal hydrolases [49]. Given that autophagy may enhance cancer cells to survive in the tumor microenvironment or prevent chronic tissue damage that can lead to cancer initiation, assessment of autophagy markers became a must in cancer research [50]. In the current study, detection of autophagosomes formation was achieved by staining with Acridine Orange, a metachromatic fluorescent dye with a suggested photosensitization effect [51]. The acidic vesicular organelles, the hallmarks of autophagy, were visualized in control and treated HCT116 cells. The combination treatment markedly elevated the amount of autophagolysosomes in the cells in comparison to individual drugs alone. In this regard, findings in the literature corroborate our results as IM promoted autophagy in CML cell lines and that was detected by acridine orange as well [36].

Unexpectedly, after we evidenced the formation of autophagosomes and autophagy activation, we evidenced increments in proapoptotic markers. After tracking the different apoptotic cascades, the apoptotic pathways were upregulated.

Colony formation assay has proved to be the method of choice to assure the cell death induced by different treatment modalities [1]. The advantage of this assay resides on testing the proliferative ability of every cell in a population to divide unlimitedly [52]. After application of a cytotoxic agent, only a little fraction of cultured cells is expected to retain the capacity to form colonies [1]. In the present study, colonies of HCT_{116} cells were subjected to combination therapy and to either drug alone; screening of the tissue growth was inspected afterwards. HCT_{116} cells were found to be sensitive to the combination treatment as compared to the lethal impact of each drug individually. In this context, IM has been reported to inhibit colorectal cancer cell growth in HCT_{116} cells [53]. In addition, colony formation assay in HCT116 cells proved the cytotoxic effect of TQ [38]. The above-mentioned data confirmed the potential cytotoxic effect of the combination studied. Consequently, a thorough investigation of apoptotic markers followed the clonogenic assay.

Bcl-2 (B-cell lymphoma 2) family of proteins, is considered of the most important proteins heavily involved in apoptosis both with pro- and antiapoptotic activities [54]. The Bcl-2 family of proteins regulates the mitochondrial apoptotic pathway [55]. A sub-group of Bcl-2 family includes BAX which is proapoptotic when overexpressed [56]. The antiapoptotic proteins include BCl-2 and BCl-XL [57]. One of the most common tactics cancer cells utilize to promote survival is antiapoptotic proteins overexpression to be a major chemoresistance factor in several human cancers [2]. Notably, the Bcl-2 gene negatively regulates two cell death pathways: apoptosis and autophagy [11]. The molecular interaction between autophagy and apoptosis relies on the mitochondria-dependent intrinsic apoptotic pathway which ends with BAX involvement [58]. On the opposite side, the upregulation of the BAX gene showed to be a negative regulator of autophagy in colorectal cancer cells [59]. Thus, targeting antiapoptotic proteins is a pharmacologic demand to reactivate the programed cell death [2]. In the current study, our combination augmented BAX, mitigated BCl-2, BCl-XL and showed significant increments in BAX/BCl-2, total, early and late apoptotic ratios in comparison to individual treatments. In this context, IM enhanced apoptosis showing relevant behavior of the markers we assessed when used alone and in combination in HCT116 cells [60]. Likewise, TQ monotherapy and in combination with other anticancer agents showed elevation in BAX levels accompanied with reduction in BCl-2 levels in three types of colon cancer cell lines [61].

Among the tumorigenesis suppression mechanisms, prostate apoptosis response-4 (Par-4), which is a proapoptotic tumor suppressor protein, exists in human beings with a pivotal role in various cancers [62]. Indeed, Par-4 expression downregulates carcinogenesis via apoptosis induction in various malignancies [63]. Notably, the overexpression of Par-4 caused significant increments in the expression of p53 and limited the long-term survival of residual cancer cells [64]. Being a physiological substrate of caspase-3; Par-4 acts as a tumor suppressor in different cancer cells keeping the normal cells intact as it does not cause apoptosis of normal or non-transformed cells on its own [65]. Herein, we hypothesized and subsequently observed Par-4 to be inversely correlated to BCl-2. In the current study, Par-4 levels showed the highest increments when HCT_{116} were

treated with our combination. In align with our results, TQ proved to elevate Par-4 levels in human malignant glioma cells [66]. Previous studies showed results in consistency with ours whereas IM significantly enhanced Par-4 expression in cells isolated from chronic lymphocytic leukemia (CLL) patients [67]. Intriguingly, analysis of the total apoptotic ratio using flow cytometry confirmed the potential apoptotic effect of the combination compared to the individual drugs.

As previously mentioned, AMPK; an autophagy inducer; plays a major role in autophagy upregulation by activating cell cycle arrest and tumor growth inhibition [68]. AMPK inhibits the mammalian target of rapamycin (mTOR) signaling pathway resulting in anticancer effect [69].

This is explained by the fact that in aggressive cancers the stress caused by treatments will increase AMPK expression [70]. Although AMPK in turn will always commence autophagy, it was also reported that in early stages of CRC tumorigenesis apoptosis is more prone to occur than autophagy [71]. This was observed by the initiation of early apoptosis, recording the highest levels by our combination. Our current work proved the potency of this pathway as the increments in AMPK level defaulted the cell to autophagy and finally invigorated apoptosis.

In-depth research studying IM-induced resistance in various types of cancers evidenced different resistance patterns evolving during IM therapy relating IM to different factors [35]. One of the main mechanisms of IM resistance is the activation of autophagy in cancer cells to face IM-induced stress and is interrelated with AMPK upregulation [44]. Additionally, IM inhibits apoptosis in early stages which is the common factor in the various resistance patterns shown in IM-treated models [43]. In the current study, the combination used proved to influence both autophagy and apoptosis whereas the rising autophagy always activated apoptosis in a stress-defense response. Indeed, the superfluous activation of proapoptotic factors, tumor suppressor Par-4, influences the different resistance patterns involved in IM resistance. The existing autophagy inhibitors, as hydroxychloroquine, have low safety profile which results in failure to achieve optimal plasma concentrations when used with tyrosine kinase inhibitors [72]. Interestingly, TQ can be considered as a natural autophagy inhibitor with potential proapoptotic, antiproliferative, antimetastatic, antioxidant and antiangiogenic effects in many cancers [73].

In conclusion, this study proved the discerned cytotoxic effect of the combination, evidenced by the inhibition of colonies formation as well as the increments of various apoptotic markers. At the same time, there was a distinct induction of autophagy which was supposed to be a tumor survival strategy. We can underscore the finding that continuous upregulation of autophagy acted as a programmed cell death technique. Indeed, both activated autophagy and apoptosis in stressed cells lead to death. AMPK, when senses the rising autophagy, it will always activate apoptosis in a stress-defense response. Our findings proved that our combination, with the mentioned doses, enhanced autophagy and apoptosis within the tumor microenvironment and explained the interrelation between them. Henceforth, we can benefit from the addition of TQ to IM to minimize the resistance cancer cells develop against IM.

Author Contributions

Dalia El-Khouly: writing – original draft, supervision, conceptualization, writing – review and editing. **Nadia A. Thabet:** conceptualization, data curation, methodology, formal analysis. **Mohamed Sayed-Ahmed:** supervision, writing – review and editing. **Mervat M. Omran:** conceptualization, data curation, writing – original draft, methodology, formal analysis, writing – review and editing.

Acknowledgments

The authors have nothing to report.

Ethics Statement

The authors have nothing to report.

Consent

The manuscript was reviewed and approved by all authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw data were generated at the National Cancer Institute, Egypt. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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