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3-Bromopyruvate potentiates TRAIL-induced apoptosis in human colon cancer cells through a reactive oxygen species- and caspase-dependent mitochondrial pathway

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Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising anticancer cytokine with minimal toxicity towards normal cells. Nevertheless, most primary cancers are often intrinsically TRAIL-resistant or can acquire resistance after TRAIL therapy. This study aimed to investigate the inhibitory effect of co-treatment of 3-Bromopyruvate (3-BP) as a potent anticancer agent with TRAIL on colon cancer cells (HT-29). The results of present study indicated that combined treatment with 3-BP and TRAIL inhibited the proliferation of HT-29 cells to a greater extent (88.4%) compared with 3-BP (54%) or TRAIL (11%) treatment alone. In contrast, the combination of 3-BP and TRAIL had no significant inhibitory effect on the proliferation of normal cells (HEK-293) (8.4%). At a cellular mechanistic level, the present study showed that 3-BP sensitized human colon cancer cells to TRAIL-induced apoptosis via ROS generation, upregulation of Bax, downregulation of Bcl-2 and survivin, release of cytochrome c into the cytosol and activation of caspase3. In normal cells, 3-BP, TRAIL or combination of both had no significant effect on the ROS levels, release of cytochrome c and caspase3 activity. Therefore, the combination of 3-BP and TRAIL can be a promising therapeutic strategy for treatment of colon cancer.

Keywords: 3-BP, TRAIL, Colorectal cancer, TRAIL resistance, Apoptosis

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths throughout the world (Favoriti et al. 2016). CRC is a disease of aging and largely affects the elderly population. Approximately 70% of cases in total develop over the age of 65 years, and about 40% of patients are over 75 years (Kim 2015). The majority of CRC cases are diagnosed at later stages (Tsikitis et al. 2014). Diagnosis of advanced CRC portends remarkably worse survival outcomes for both younger and older adults (Tannenbaum et al. 2014). Chemotherapy is the main treatment for recurrent colon cancer, but this therapy has severe systemic side effects. Moreover, about 50% of patients with colon cancer, die from subsequent metastatic disease (Arnold et al. 2017; Favoriti et al. 2016). Therefore, there is an urgent need to develop a new strategy for treatment of colon cancer. Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL/Apo2L) is a member of the TNF gene superfamily involved in cancer biology since its discovery in 1995. TRAIL has been shown to have potent antiproliferative activity in many types of cancer cells and low cytotoxicity in normal cells (El Fajoui et al. 2011; Mérimo et al. 2007; Tafuku et al. 2006). TRAIL induces apoptosis by interacting with death receptors in cancer cells (Almasan and Ashkenazi 2003). It binds to five distinct receptors (DcR1, DcR2, DR4 DR5, and OPG) on the cell surface, two of which are death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), three of which are decoy receptors, DCR1 (TRAIL-R3), DCR2, (TRAIL-R4) and osteoprotegerin (OPG) (Almasan and Ashkenazi 2003; Van Geelen et al. 2004). Binding of TRAIL to the death receptors 4 and 5 leads to the formation of the death-inducing signaling complex (DISC) and activation of caspase 8 (Carlo-Stella et al. 2007). Active caspase-8 cleaves and activates effector caspases such as caspase-3. In type I cells, this pathway is sufficient to cause cell death. In contrast, in type II cells, involvement of the mitochondrial pathway (intrinsic

pathway) is required. Caspase-8 cleaves Bid into its active form t-Bid, which translocates to the outer mitochondrial membrane and causes release of cytochrome c and other apoptotic proteins to the cytoplasm, activation of caspase-9, caspase-7, caspase-3, and finally apoptosis (Carlo-Stella et al. 2007). Caspases are a family of protease enzymes playing essential roles in programmed cell death. Caspase-3 acts as a major executioner protein in proteolytic degradation during apoptosis (Huang et al. 2018). Studies have indicated that the activation of the apoptosis effector caspase-3 is essential for TRAIL induced apoptosis in human colon adenocarcinoma (Kauntz et al. 2012). Despite many study showing the effective anti-cancer activities of TRAIL, a number of cancer cells are resistant to TRAIL-induced apoptosis (Stolfi et al. 2012). The mechanisms involved in TRAIL resistance, including mutation of caspase 8 or alternations in expression of the Bcl-2 family proteins, downregulation of TRAIL receptors, overexpression of caspases inhibitors such as cFLIP, X-linked inhibitor of apoptosis protein (XIAP) and survivin (Zhang and Fang 2005). Survivin is a member of the inhibitor of apoptosis protein (IAP) family, which is frequently highly expressed in cancer cells (Park et al. 2013). Survivin prevents the activation of caspase-3 and caspase-9 and inhibits both the extrinsic and intrinsic apoptotic pathways simultaneously (Deveraux and Reed 1999). It is up-regulated in colorectal cancer and thought to represent a major hurdle for TRAIL-based therapies in colon cancer (Kim et al. 2003). Several studies have reported that TRAIL resistance can be overcome by combined treatment with chemotherapeutic agents and TRAIL (El Fajoui et al. 2011; Zhu et al. 2004). 3-Bromopyruvate (3-BP) as a hexokinase II inhibitor has shown to induce cell death in colon cancer cells (HT-29) through a variety of different mechanisms (Ko et al. 2004; Tang et al. 2012). Two previous studies have demonstrated that 3-BP enhances TRAIL-induced apoptosis in nasopharyngeal and breast cancer cells (Can et al. 2017; Chen et al. 2018). It has not been examined whether co-treatment of 3-BP and TRAIL

can attenuate TRAIL resistance in colon cancer cells. Therefore, we aimed to investigate whether 3-BP potentiates the apoptosis induced by TRAIL in HT-29 cells. The findings of the present study revealed that the co-treatment of 3-BP and TRAIL could significantly sensitize colon cancer cells to TRAIL. In this study we reported the potential mechanisms underlying the cooperative induction of apoptosis by combination of 3-BP and TRAIL in HT-29 cells.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM) was obtained from Applichem (Germany). Penicillin-streptomycin, trypsin, and fetal bovine serum (FBS) were purchased from Gibco (USA). Dimethyl sulfoxide (DMSO), phosphate buffer saline (NaCl, KCl, Na₂HPO₄(2H₂O), KH₂PO₄), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and rhodamine 123 were obtained from Sigma-Aldrich. FlowCelect annexin red kit was purchased from Millipore (Germany). Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (London, UK).

Cell Culture

HT-29 (human colorectal adenocarcinoma) and HEK-293 (Human embryonic kidney) cell lines were purchased from the Pasture Institute, (Tehran, Iran). The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂ (Song et al. 2005).

Cell viability assay

Cell viability was determined by the MTT assay. Briefly the cells were seeded on 96-well plates (7×10^3 cells/well) and treated with different concentrations of 3-BP (40, 80, 160, and 320 µM), TRAIL (50, 100, 200 and 400 ng/ml) or combination of them for various time periods (24, 48, 72

h). Afterwards, 20 μ L of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 4 h at 37 °C under 5% CO₂. The medium was then replaced with 150 μ l dimethyl sulfoxide (DMSO) per well to solubilize the formazan crystals. Finally, the absorbance was measured at 570 nm using a microplate reader (Biotek, H1M, USA) (Abbaszadeh et al. 2014).

Evaluation of apoptosis by annexin V and 7-amino-actinomycin D (7-AAD) staining

Enumeration of apoptotic cells was carried out using annexin V apoptosis detection kit with 7-amino-actinomycin D (7-AAD) according to the manufacturer's instruction. Briefly, the cells were seeded into 12-well plates at a density of 5×10^5 cells/ml and treated with 3-BP, TRAIL or combination of both for 48 h. The cells were washed twice with cold PBS, resuspended in 500 μ l binding buffer (BD Pharmingen) and stained with annexin VCF647 and 7-AAD (a nucleic acid dye for the exclusion of nonviable cells) for 15 min at room temperature in the dark. After addition of binding buffer to each tube the stained cells were analyzed by flow cytometry within 1 h (Kang et al. 2014).

Determination of intracellular ROS level

Reactive oxygen species (ROS) levels were measured by fluorometry. Briefly, the cells were seeded in 24-well plates at a density of 1×10^4 , allowed to attach overnight and treated with BP, TRAIL or combination of them for 4-48 hr. After washing with PBS the cells were stained with 20 μ l of DCFDA at 37 °C for 30 min. The cells were harvested and intensity of fluorescence was measured using a fluorometer (Biotek, H1M, USA) at an excitation wavelength of 488 nm and an emission wavelength of 528 nm (Carrasco-Torres et al. 2017).

In order to determine the involvement of ROS in HT-29 cells apoptosis induced by 3-BP, TRAIL or combination of them, the cells were pretreated with ROS scavenger N-acetylcysteine (NAC)

and glutathione and the influence of NAC and glutathione on HT-29 cells was determined by MTT assay and DCFDA staining.

Measurement of mitochondrial membrane potential (MMP)

MMP was estimated by a fluorescent tracer dye, Rhodamine 123, which is widely used to detect the mitochondrial depolarization depending on the reduced fluorescence. Briefly, after treatment, the cells were washed twice with PBS and incubated with rhodamine 123 (0.1 µg/mL) at 37°C in dark for 30 min. The cells were then washed twice with PBS and resuspended in 1000 µL PBS. Finally the cells were analyzed using flow cytometry at 488 nm and 520 nm for the excitation and emission wavelength respectively (Zhu et al. 2015).

Determination of caspase-3 activity

Enzymatic activity of caspases 3 was measured using a colorimetric assay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). In brief, the cells were treated, harvested, and lysed with lysis buffer (25 mmol/L HEPES, 5 mmol/L MgCl₂, 5 mmol/L EDTA, 2 mmol/L DTT, 0.1% CHAPS) for 30 min. The lysates were centrifuged and 100 µg of the protein was incubated with a substrate reaction buffer containing a caspase-3 colorimetric substrate (acetyl-Asp-Glu-val-Asp p-nitroanilide) at 37°C for 2 h. Release of pNA was quantified spectrophotometrically at a wavelength of 405 nm (Ma et al. 2017).

Cytochrome c release assay

The concentration of released cytochrome c from mitochondria was determined using the quantikine cytochrome c immunoassay kit obtained from the R&D systems (Minneapolis, MN, USA). Cytochrome c release assay was performed according to the manufacturer's protocol (Wu et al. 2016).

RNA extraction and cDNA synthesis

Total RNA was isolated from HT-29 cells using RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from total RNA using QuantiTect® reverse transcription kit (Qiagen) and oligo(dT) 17-24 primers (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instruction.

Quantitative real-time polymerase chain reaction (qRT-PCR)

In order to quantify the amount of mRNA in untreated and treated cells, quantitative real-time PCR was carried out via Rotor-Gene Q apparatus (Qiagen, Germany) according to the manufacturer's protocol. A PCR reaction mixture of 25 µl containing 12 µL of SYBR Green I PCR Master Mix (TaKaRa, Japan), 1 µL of forward and reverse oligonucleotide (400 nM), cDNA template (300 ng) and ddH₂O was used. Four pairs of primers were separately used: three pairs to amplify, bcl2, bax, survivin genes, the other pair for the endogenous control gene, GAPDH. Primers used are presented in Table 1. All the samples were run in triplicate. Cycling conditions were as follows: 15 min at 50 °C for cDNA synthesis, 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C to denature the DNA, and 45 sec at 60 °C to anneal and extend the template. After 40 cycles, the samples were run for the dissociation protocol (i.e. melting curve analysis). Gene expression was determined as the relative fold change using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers were synthesized by Pishgam Company (IRAN) (Porameesanaporn et al. 2013).

Statistical analysis

The results were expressed as mean ± SEM. The statistical analysis was carried out using One-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

3-BP enhances TRAIL- mediated apoptosis in HT- 29 cells

MTT assay was used to evaluate the effect of 3-BP on TRAIL-induced apoptosis in HT-29 cells. The cells were treated with concentrations of 40, 80, 160, and 320 μM 3-BP and 50, 100, 200 and 400 ng/ml TRAIL for 24, 48 and 72. As shown in Fig. 1, 3-BP and TRAIL inhibited cell proliferation. Based on these preliminary data, the concentrations of 120 μM 3-BP and 100 ng/ml TRAIL were selected using MTT assay for the next studies. The cells were exposed to 3-BP (120 μM) with or without TRAIL (100ng/ml) for 48 h. The results of the MTT assay indicated that 3-BP (120 μM) alone significantly inhibited HT-29 cell viability ($p < 0.01$), while TRAIL (100 ng/ml) alone had a minor inhibitory effect on the proliferation of colon cancer cells. The combination treatment with 3-BP (120 μM) and TRAIL (100 ng/ml) inhibited colon cancer cell viability to a greater extent (88.4%) compared with 3-BP (54%) or TRAIL (11%) treatment alone ($p < 0.01$ and $p < 0.001$ respectively). In contrast, the combination of 3-BP and TRAIL inhibited normal cell (HEK-293) viability by 8.4% (Fig. 2). Therefore, the results showed that co-treatment with 3-BP and TRAIL synergistically induced apoptosis in colon cancer cells with little or no toxicity against normal cells.

3-BP significantly triggers TRAIL- induced apoptosis in HT-29 cells

Annexin VCF647 / 7AAD flow cytometric was used to determine the percentage of apoptotic cells induced by 3-BP (120 μM), TRAIL (100 ng/ml) or a combination of both in HT-29 cells. Flow cytometry investigation revealed that the population of early apoptotic cells (Annexin V-positive, 7-AAD -negative) at 48 hours after treatment with 3-BP or TRAIL was approximately 19.80 ($p < 0.01$) and 0.1% respectively. (Fig. 3 and 4). Co-treatment of 3-BP and TRAIL significantly increased early apoptotic cells to 43.4% ($p < 0.001$). The population of viable cells (Annexin V-

negative and 7-AAD -negative) significantly decreased at 48 hours after co-treatment with 3-BP and TRAIL compared with TRAIL treatment alone ($p < 0.001$). These data indicate that 3-BP enhances TRAIL-induced apoptosis in TRAIL-resistant HT-29 cells.

Potentialiation of TRAIL-induced apoptosis by 3-BP is ROS-dependent

In order to determine whether 3-BP mediates its anticancer effects through ROS generation, intracellular ROS levels were measured by fluorimetry using the fluorescent probe DCF-DA. The results demonstrated that intracellular ROS levels were significantly increased in colon cancer cells after treatment with 3-BP, TRAIL or combination of them for 12 ($p < 0.001$) and 24 h ($p < 0.01$). In normal cells, the ROS levels were not significantly elevated by 3-BP, TRAIL or combination of both (Fig. 5). In this study effect of ROS on the enhancement of TRAIL-induced apoptosis by 3-BP was evaluated. As shown in Fig. 6, pretreatment with ROS scavenger N-acetylcysteine and glutathione considerably reduced the potentiation of TRAIL-induced apoptosis by 3-BP in HT-29 cells ($p < 0.001$). These results suggest that ROS play an important role in mediating the effects of 3-BP on TRAIL-induced apoptosis. Therefore ROS induction can be a primary mechanism responsible for the synergistic anti-cancer activity of the combination of 3-BP and TRAIL.

Induction of mitochondrial membrane potential loss by 3-BP contributes to synergistic interaction with TRAIL

Mitochondria play a central role in activating apoptosis in mammalian cells through the release of pro-apoptotic proteins contained in the intermembrane space. MMP was measured by a fluorescent dye, Rhodamine 123. In this study 3-BP significantly induced the collapse of MMP in colon cancer cells ($P < 0.01$). In contrast, TRAIL alone had no significant effect on the loss of MMP in HT-29

cells. Combined treatment with 3-BP and TRAIL caused a significant loss of MMP in HT-29 cells compared with 3-BP or TRAIL treatment alone ($p < 0.01$ and $p < 0.001$ respectively) (Fig. 7 and 8). These results suggest that the mitochondria-dependent apoptosis pathway is involved in colon cancer cells death by co- treatment of 3-BP and TRAIL.

Release of cytochrome c by 3-BP contributes to synergistic anticancer effects of TRAIL

During apoptotic cell death, the early events that occur are mitochondrial depolarization and loss of cytochrome c from the mitochondrial intermembrane space. In order to find the role of mitochondria in induced apoptosis by 3-BP, TRAIL or both of them, the release of cytochrome c from mitochondria into the cytosol was tested in HT-29 cells. The results indicated that 3-BP or TRAIL alone increased the content of cytosol cytochrome c in HT-29 cells ($p < 0.01$). In this study cytochrome c was significantly released into the cytosol of HT-29 cells by the combination of 3-BP and TRAIL compared with 3-BP or TRAIL alone ($p < 0.01$ and $p < 0.001$ respectively) (Fig. 9). In normal cells, 3-BP, TRAIL or combination of them had no significant effect on the release of cytochrome c. These data suggest that the combination of 3-BP and TRAIL induces apoptosis in colon cancer cells in a synergistic fashion through the release of cytochrome c.

3-BP enhances TRAIL-induced apoptosis through caspase activation

Caspases are a family of protease enzymes that play an critical role in programmed cell death. Caspase-3 is the major effector caspase that interacts with caspase-8 and caspase-9. As shown in Fig. 10, treatment with TRAIL or 3-BP alone affected the caspases 3 activation in HT-29 cells ($p < 0.01$). Combined treatment with TRAIL and 3-BP significantly increased caspase-3 activity in colon cancer cells compared with 3-BP or TRAIL treatment alone ($p < 0.01$ and $p < 0.001$ respectively). In normal cells, 3-BP, TRAIL or combination of them had no effect on caspase-3

activity. These findings showed that combined treatment of 3-BP with TRAIL induced apoptotic death in HT-29 cells through a caspase dependent pathway.

3-BP enhances TRAIL- induced apoptosis through upregulation of Bax and downregulation of Bcl-2 and survivin

To determine the molecular mechanisms responsible for apoptosis induced by the combination of 3-BP and TRAIL, the expression level of several pro-apoptotic and anti-apoptotic genes was evaluated. Real time RT-PCR was carried out to measure the mRNA expressions of Bax, Bcl-2, and survivin in HT-29 cells treated with 3-BP, TRAIL or combination of them. The Real time RT-PCR data analysis revealed that Bcl-2 and survivin genes expression was significantly down-regulated in HT-29 cells following co-treatment with 3-BP and TRAIL compared with 3-BP or TRAIL treatment alone ($p < 0.01$ and $p < 0.001$ respectively). The expression of Bax gene was significantly up-regulated by the combination of 3-BP and TRAIL in HT-29 cells compared with 3-BP or TRAIL treatment alone ($p < 0.01$ and $p < 0.001$ respectively). TRAIL alone had no significant effect on the up-regulation of Bax and downregulation of Bcl-2 and survivin in colon cancer cells. (Fig. 11).

The ratio of Bcl-2 to Bax (Bcl-2 / Bax) for 3-BP, TRAIL and combination of both was estimated as 0.178, 0.607 and 0.087 respectively.

These results indicate that upregulation of pro-apoptotic gene Bax and downregulation of anti-apoptotic gene Bcl-2 and survivin are involved in the mechanism by which 3-BP enhances TRAIL-induced apoptosis.

Discussion

TRAIL is a promising anticancer agent which has been shown to selectively induce apoptosis in many types of cancer cells and not in most normal cells (Stuckey and Shah 2013). However,

resistance to TRAIL in multiple cancer cells could limit its clinical application for treatment of malignant tumors. The molecular basis of resistance to TRAIL-induced apoptosis is complex and multifactorial (Zhang and Fang 2005). To overcome TRAIL resistance, developing strategies and understanding the mechanisms underlying such resistance are necessary for the use of TRAIL in cancer therapy. It has been reported that the combination of TRAIL with a variety of chemotherapeutic agents can sensitize resistant cancer cells and result in enhanced anti-cancer activity (El Fajoui et al. 2011; Zhu et al. 2004). 3-Bromopyruvate (3-BP) is a Potent anticancer alkylating agent which exerts its anti-cancer influence via different molecules and pathways, including inhibition of key glycolysis enzymes (hexokinase II and lactate dehydrogenase), inhibition of mitochondrial oxidative phosphorylation, angiogenesis, and energy production in cancer cells. 3-Bromopyruvate (3-BP) also induces cancer cell death through hydrogen peroxide generation (oxidative stress effect) (Ho et al. 2016; Schaefer et al. 2012). 3-BP has been shown to have potent synergistic effects with various anti-cancer drugs in vitro and in vivo (Chong et al. 2017). The present study showed for the first time that 3-BP enhanced TRAIL-induced apoptosis in colon cancer cells. Previous studies have demonstrated that 3-BP sensitizes nasopharyngeal and breast cancer cells to TRAIL (Can et al. 2017; Chen et al. 2018). In this study TRAIL alone had no significant antiproliferative effect on colon cancer cells. The combination of 3-BP and TRAIL significantly inhibited the proliferation of HT-29 cells compared with 3-BP or TRAIL treatment alone. These findings show that HT-29 cells are resistant to TRAIL and are consistent with the results of previous studies (Lee et al. 2011; Park et al. 2012). TRAIL induces apoptosis in various types of cancer cells in vitro and in vivo, but has little or no toxicity against normal cells (Li et al. 2003). 3-BP has shown to be less toxic to normal cells both in vitro and in vivo (Buijs et al. 2009; El Sayed et al. 2014). The present study indicated that the combination of 3-BP and TRAIL had

no significant antiproliferative effect on normal cells. Bcl-2 family proteins are important mediators of cell death and survival, which are classified into two subgroups: the anti-apoptotic members (Bcl-2 and Bcl-xl) and pro-apoptotic members (Bax, Bad and Bid) (Shamas-Din et al. 2013). These proteins control programmed cell death by regulation of outer mitochondrial membrane permeabilization. The anti-apoptotic Bcl-2 proteins directly interact with pro-apoptotic proteins and inhibit their activity and apoptosis. Pro-apoptotic proteins such as Bax and Bak directly permeabilize the mitochondrial outer membrane that lead to cytochrome c release during apoptosis (Czabotar et al. 2014). Overexpression of anti-apoptotic proteins Bcl-2 and Bcl-xL have been found to be associated with TRAIL resistance in cancer cells (Fulda et al. 2002; Song et al. 2007). In the present study, upregulation of Bax and downregulation of Bcl-2 were not significantly affected by TRAIL alone in colon cancer cells. While the combination treatment of 3-BP and TRAIL significantly upregulated Bax and downregulated Bcl-2 expression level in HT-29 cells. Therefore, upregulation of pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 can be the mechanisms of enhancement of TRAIL-induced apoptosis by 3-BP. Survivin, is a member of the inhibitor of apoptosis (IAP) protein family that prevents caspase activation and inhibits apoptosis. It is overexpressed in most human cancers and promotes tumorigenesis (Altieri 2010; Dohi et al. 2004). Survivin has been reported to be involved in cancer cell resistance to TRAIL (Azuhata et al. 2006). In this study, co- treatment of 3-BP and TRAIL significantly inhibited the expression of survivin. Therefore, inhibition of survivin can be involved in the mechanism by which 3-BP enhances TRAIL-induced apoptosis. This study showed that the co-treatment of 3-BP and TRAIL significantly induced loss of mitochondrial membrane potential (MMP) and increased the release of cytochrome c into the cytosol in HT-29 cells. In normal cells, 3-BP, TRAIL or combination of both had no significant effect on the release of cytochrome c. The loss of MMP

and release of cytochrome c lead to the activation of caspase-3 and subsequent apoptosis (Gupta et al. 2009). Most signaling pathways activated by anticancer agent ultimately lead to the activation of caspases, a family of cysteine proteases that play an essential role in programmed cell death (Choi et al. 2009). The effector caspase-3 activation has been shown to be involved in TRAIL-induced apoptosis (Shao et al. 2005). The results of this study indicated that the combination of 3-BP and TRAIL significantly increased caspase 3 activity compared with 3-BP or TRAIL alone . In normal cells, the caspase3 activity was not significantly affected by the combination of 3-BP and TRAIL. These data suggest that 3-Bromopyruvate sensitizes human colon cancer cells to TRAIL-induced apoptosis through a caspase dependent mitochondrial pathway. Reactive oxygen species (ROS) are a group of highly reactive ions and molecules derived from molecular oxygen (O₂). A large number of anticancer agents effectively kill cancer cells and overcome drug resistance through enhancing ROS generation (Zhang et al. 2015). Several anticancer drugs have been found to enhance the expression of TRAIL receptors (DR5) through ROS generation and sensitize cancer cells to TRAIL induced-apoptosis (Jung et al. 2005; Kim et al. 2006; Kwon et al. 2008). In this study 3-BP, TRAIL or combination of both induced ROS generation in colon cancer cells. In normal cells, 3-BP, TRAIL or combination of both had no significant effect on the ROS levels. Quenching of ROS by N-acetylcysteine and glutathione remarkably reduced the enhancement of TRAIL-induced apoptosis by 3-BP in HT-29 cells. These results demonstrate that combined treatment of 3-BP with TRAIL attenuates TRAIL resistance in colon cancer cells through a ROS dependent pathway. Taken together, the present study indicated that 3-BP sensitized HT-29 cells to TRAIL-induced apoptosis through a ROS-and caspase-dependent mitochondrial pathway with little or no toxicity against normal cells. Therefore the combination of 3-BP and TRAIL can be an effective therapeutic strategy for treatment of colorectal cancer.

Further studies are needed to evaluate the combination of 3-BP and TRAIL for treatment of colon cancer in vivo.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure 1. Inhibitory effect of 3-BP or TRAIL on HT-29 cells. Cells were treated with 3-BP (0-320 μ M) or TRAIL (0-400 ng/ml) . Cell viability was measured by MTT assay for 24, 48 and 72 hr. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: $p<0.05$, **: $p<0.01$, *** $p<0.001$ compared with control group.

Figure 2. 3-BP enhanced TRAIL-mediated apoptosis in HT- 29 cells. Effect of 3-BP , TRAIL or combination of them on cell viability in HT- 29 and Normal cells. Cells were treated with 3-BP (120 μ M), TRAIL (100 ng/ml) or combination of them . Cell viability was measured by MTT assay for 48 hr. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: $p<0.01$, **: $p<0.001$ compared with control group. #: $P<0.01$ compared with 3-BP group. &: $p<0.001$ compared with TRAIL group.

Figure 3. 3-BP significantly trigger TRAIL- induced apoptosis in HT29 cells. Cells were treated with 3-BP (120 μ M) , TRAIL(100 ng/ml) or combination of them for 48 h. Cell death was measured using annexinV/ 7-AAD staining and flow cytometry for 48h. A: Untreated cells, B:3-BP, C: TRAIL, D: 3-BP+ TRAIL.

Figure 4. The percentage of cellular apoptosis (A) and necrosis (B) was determined using annexin V/7-AAD staining and flow cytometry in HT-29 cells treated with 3-BP, TRAIL or combination of them for 48 h. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: $p<0.01$, **: $p<0.001$ compared with control group. #: $P<0.01$ compared with 3-BP group. &: $p<0.001$ compared with TRAIL group.

Figure 5. Potentiation of TRAIL-induced apoptosis by 3-BP is ROS-dependent. The ROS level was measured after treatment with BP, TRAIL or combination of them for 4 (A), 12(B), 24(C) and 48 h(D) in HT-29 and normal cells. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: $p<0.01$, **: $p<0.001$ compared with untreated HT- 29 cells (control group) . #: $p<0.01$, ##: $p<0.001$ compared with untreated normal cells (control group).

Figure 6. pretreatment with ROS scavenger N-acetylcysteine and glutathione considerably reduced the potentiation of TRAIL-induced apoptosis by 3-BP in HT-29 cells. Effect of N-acetylcysteine (NAC) and glutathione on cell death after 48h treatment with 3-BP , TRAIL or combination of them. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: $p<0.01$, **: $p<0.001$ compared with control group.

Figure 7. Induction of mitochondrial membrane potential loss by 3-BP contributes to synergistic interaction with TRAIL. Mitochondrial membrane potential was measured using fluorescent dye rhodamine 123 staining and flow cytometry in HT-29 cells treated with 3-BP, TRAIL and combination of them for 48h. A: Untreated cells. B: 3-BP, C: TRAIL, D: 3-BP+ TRAIL.

Figure 8. Mitochondrial membrane potential in HT-29 cells after 48h treatment with 3-BP, TRAIL and combination of them. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: p<0.001 compared with control group. #: P<0.01 compared with 3-BP group. &: p<0.001 compared with TRAIL group.

Figure 9. Release of cytochrome c by 3-BP contributes to synergistic anticancer effects of TRAIL. Release of cytochrome c into the cytosol in HT-29 and normal cells after 48h treatment with 3-BP, TRAIL and combination of them. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: p<0.01, **: p<0.001 compared with control group. #: P<0.01 compared with 3-BP group. &: p<0.001 compared with TRAIL group.

Figure 10. 3-BP enhances TRAIL-induced apoptosis through caspase activation. Activity of caspase-3 in HT-29 and normal cells after 48h treatment with 3-BP, TRAIL and combination of them. Values are presented as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: p<0.01, **: p<0.001 compared with control group. #: P<0.01 compared with 3-BP group. &: p<0.001 compared with TRAIL group.

Figure 11. 3-BP Enhances TRAIL-induced apoptosis through upregulation of Bax and downregulation of Bcl-2 and survivin. The Expression levels of Bax, Bcl-2 and survivin in HT-29 cells treated with 3-BP, TRAIL or a combination of them. Values are expressed as mean $\pm$ SD of 3 separate triplicated determinations (n=9). *: p<0.01, **: p<0.001 compared with control group. #: P<0.01 compared with 3-BP group. &: p<0.001 compared with TRAIL group.

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Table 1. Oligonucleotide Sequences for Interest and Reference Genes

Oligo Name	Sequence (5' → 3')	Amplicon, bp
gapdh-F	AAGTTCAACGGCACAGTCAAGG	22
gapdh-R	CATACTCAGCACCAGCATCACC	22
bax-F	AGGGTGGCTGGGAAGGC	17
bax-R	TGAGCGAGGCGGTGAGG	17
bcl2-F	ATCGCTCTGTGGATGACTGAGTAC	24
bcl2-R	AGAGACAGCCAGGAGAAATCAAAC	24
Survivin-F	CCCTTTCTCAAGGACCACCG	20
Survivin-R	TGTCCTCTCTGGGGTCGTCA	21

Draft