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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

The combination of Bleomycin with TRAIL agonists or PKC inhibitors sensitizes solid tumor cells to BLM-mediated apoptosis: new strategies to overcome chemotherapy resistance of tumors

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Abstract In this study we evaluated the effects of low dose of bleomycin in an associative treatment strategy in solid tumor cells. For this purpose, Human and murine colon cancer (SW480, HCT8, and CT26), and murine melanoma (B16-F10) cells were treated with different agents including protein kinase C, and c-jun NH2-terminal kinase inhibitors, and tumor necrosis factor-related apoptosis-inducing ligand. Apoptosis was identified by morphological criteria. Reactive oxygen species are evaluated by flow cytometry. Our data showed that bleomycin (100 µM) induced apoptosis in all the four cell lines tested with a level ranging from 30 to 60%. However, at lower dose (25 µM), bleomycin was less efficient to trigger apoptosis. In contrast, when bleomycin (25 µM) was combined with the protein kinase C inhibitor chelerythrine, or tumor necrosis factor-related apoptosis-inducing ligand, it elicited more apoptotic cell death ranging from 40 to 75%, depending on the cell type, whereas when it was associated with the c-jun NH2-terminal kinase inhibitor SP600125, bleomycin displayed different cell death responses. If bleomycin and SP600125 enhanced apoptosis in two colon cancer cells, HCT-8, and CT26, they reduced to 50% apoptosis in the melanoma B16-F10 cells, and were not synergistic in the human colon cancer cells, SW480. This synergism seemed to rely partially to reactive oxygen species, because *N*-acetyl cysteine inhibited apoptosis in some cells and with some agents. These findings indicate that tumor necrosis factor-related apoptosis-inducing ligand, and protein kinase C inhibition can represent candidates for improved cancer chemotherapy.

Keywords Bleomycin · Solid tumor cell lines · Apoptosis · PKC · TRAIL · ROS

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Introduction

Bleomycin (BLM), an anticancer drug, is used clinically in combination chemotherapy against certain types of lymphomas (Bayer et al. 1992; Bonadonna et al. 1972), squamous-cell carcinomas (Hamakawa et al. 1998), and germ-cell tumors (Levi et al. 1993). The mechanism of the antineoplasic effect of BLM is related to its ability to link several metals including iron, forming a complex which reduces molecular oxygen to superoxide and hydroxyl radicals that can cause single-stranded and double-stranded DNA cleavages (Iqbal et al. 1976; Sausville et al. 1978). The extent of the DNA cleavage was dependent on the BLM concentration and time of incubation (Iqbal et al. 1976; Moore et al. 1985).

BLM is an attractive drug, as it exhibits low myelosuppression (Boggs et al. 1974) and low immunosuppression (Lehane et al. 1975). However, because BLM induces fibrosis, therapeutic efficacy is limited by its dose-dependent lung toxicity (Martin et al. 2005; Sleijfer 2001). At low doses of BLM, the incidence of pulmonary fibrosis was low indeed absent. At high doses, BLM causes extensive DNA breaks and rapidly, initiating an apoptotic

process (Tounekti et al. 2001). Apoptosis induction by BLM has been observed in various cell types particularly alveolar epithelial cells (Lee et al. 2005; Wallach-Dayan et al. 2006). Some data studies have suggested that extensive apoptosis in the alveolar epithelial cells is an important determinant in the pathogenesis of pulmonary fibrosis (Jin and Dong 2011; Hagimoto et al. 1997; Wang et al. 2000). In another hand, the induction of lung fibrosis in patients exposed to BLM (Duggan et al. 2003; Hagimoto et al. 1997) was also related to the ability of the molecule to generate free radicals such as reactive oxygen species (ROS) (Hagiwara et al. 2000; Wallach-Dayan et al. 2006).

Recently, a new type of cancer treatment has been introduced that combines anticancer drugs with agents such as mitogen activated protein kinase (MAPK) inhibitors (Benhar et al. 2001; Fujiwara et al. 2007; Roudier et al. 2006; Zhang et al. 2015) or protein kinase C (PKC) inhibitor (Basu et al. 1991; Mitsiades et al. 2002). These combinations increase cytotoxicity of some anticancer drugs and reduce systemic drug dosage without decreasing efficacity. In another hand, many studies have demonstrated that inhibitors of MAPK provide protection against inflammation and fibrosis in fibrablasts cells lines (Lim et al. 2014; Rebeyrol et al. 2012).

In order to reduce pulmonary fibrosis caused by high doses of BLM we investigated in this study, the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), PKC, and c-jun NH2-terminal kinase (JNK) inhibitors on cell sensitivity to BLM-induced cell death by using a low dose of this drug.

Materials and methods

Drugs and reagents

Lyophilized BLM (kindly provided by Nippon Kayaku (Tokyo, Japan)) was dissolved in sterile water and stored at −20 °C. The human recombinant TRAIL (hrTRAIL) was produced and used as described previously (Schneider 2000). JNK1,-2,-3 and PKC inhibitors (iPKC) are from Biomol (tebu-bio, PA), and all other chemicals and reagents are from Sigma (Saint Quentin Fallavier, France).

Cell culture

The human colon carcinoma cell lines SW480 and HCT-8 were purchased from American Tissue Culture Collection (Manassas, VA). All cell lines were grown in 1:1 (v/v) DMEM: HAM-F10 (Biowhittaker, Fontenay-sous bois, France), supplemented with 5% FCS (Gibco BRL, Eriny, France), and 2 mmol/L L-glutamine at 37 °C in a humidified atmosphere. Cells were routinely passaged with 0.125% trypsin-0.1% EDTA, and were washed once in the culture medium before treatment. For B16-F10 murine melanoma cells and CT-26 murine colon adenocarcinoma cells, purchased from American Tissue Culture Collection (Manassas, VA), cells were cultured in RPMI 1640 medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum and L-glutamine (2 mM) in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Apoptosis detection

Apoptosis was assessed by staining the nuclear chromatin with Hoechst 33342 dye. Briefly, untreated and treated cells (2×10^5/mL) were collected, washed with PBS, stained with 1 µg/mL Hoechst 33342 dye for 15 min at 37 °C, mounted on glass slides, and observed under microscope. The percentage of apoptotic cells (chromatin condensation and nuclear fragmentation) was determined by counting 300 cells in each sample.

Flow cytometry studies

Cells were pretreated 60 min with the different agents as indicated and incubated for 48 h in the presence or absence of 25 µM BLM. ROS measurements was assessed in the presence of 6.6 µM dihydro-ethidium (Sigma-Aldrich) for 15 min at 37 °C and analyzed by flow cytometry as described (Brahim et al. 2007).

Results and discussion

Induction of apoptosis in cancer cell lines of different origins by BLM

We have previously observed that BLM induced apoptosis in leukemia and oral cancer cell lines (Brahim et al. 2007, 2008). To verify the proapoptotic effect of this drug in other cancer cells from other origins, we analyzed the induction of apoptosis in human and murine colon cancer cells (SW480, HCT-8, and CT26), and murine melanoma (B16-F10) cells. Following a 48-h incubation with 100 µM BLM, the four cell lines tested displayed typical morphologic features of apoptotic cells with chromatin condensation and nuclei fragmentation ranging from 40 to 90%, as visualized by fluorescence microscopy after DNA staining with Hoechst 33342 (Fig. 1).

Synergy of BLM and iPKC

Since BLM-induced apoptosis was obtained at high concentration of the drug (100 µM), we investigated whether BLM was able to induced apoptosis at low doses but in

combination with molecules that are known to regulate cell apoptosis (Brahim et al. 2008). We therefore investigated the role of PKC in BLM-induced apoptosis. Incubation of cells with chelerythrine, a broad spectrum iPKC, enhance the number of cells with condensed chromatin and nuclei fragmentation triggered by 25 µM BLM (Fig. 2). The increase of the percentage of apoptotic cells induced by chelerythrine varied between 40 and 60% dependent of the cell type. These results indicate that BLM is able to induce apoptosis in the four cell line tested at a dose four times lower than that when it used alone.

Synergy of BLM and JNK inhibitor

Modulation of the activity of JNK, a member of the MAPK, can either promote or inhibit apoptotic processes, depending on cell system and contexts. To investigate whether JNK was involved in BLM-induced apoptosis in cancer cells, we analyzed the effect of JNK inhibition of the apoptotic activity of the drug. As shown in Fig. 3, following a 48-h incubation with BLM (25 µM) and the JNK inhibitor SP600125 (5–10 µM), the four cell lines displayed different responses. In the human colon cancer cells SW480, SP600125 did not modify BLM-induced apoptosis. However, JNK inhibition sensitized the two other colon cancer cells (HCT-8, and CT26) to BLM-induced apoptosis. In contrast, the addition of SP600125 to cell cultures reduced to 50% the apoptosis triggered by BLM in the melanoma B16-F10 cells. These results provide evidence attesting that JNK activation can exert pro-apoptotic or anti-apoptotic effect depending on cell type.

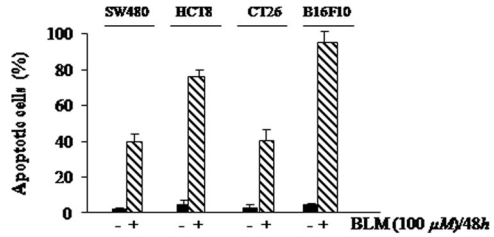

Fig. 1 BLM induced apoptosis in various cancer cell lines. The human SW480 and HCT-8, and the murine CT26 colon cancer cells, and the melanoma B16-F10 cancer cells were treated either with 100 µM of BLM for 48 h. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye. In untreated cells, the percentage of apoptosis was always lower than 5%. Data represent mean ± SD (*bars*) of at least three independent experiments

Synergy of BLM and TRAIL

To further explore the antineoplastic activity of BLM, we investigated its propapototic effect in combination with TRAIL, a molecule that induces apoptosis in a vast series of different neoplasia (Debatin and Krammer 2004). We then investigated whether hrTRAIL may affect BLM-induced apoptosis. When different cell lines were incubated with 25 µM BLM for 42–45 h and with additional treatment for 3 or 6 h with 500 nM hrTRAIL, more pronounced apoptotic responses ranging from 40 to 60% were observed in all cell lines (Fig. 4). The additional 3 h treatment with hrTRAIL (for B16-F10 and CT26 cells) instead 6 h (for SW480 and HCT8) is due to the fact that B16-F10 and CT26 cells are more sensitive to hrTRAIL alone than the two other cell lines (data not shown). These results indicate that TRAIL also contributed to the enhancement of BLM-induced apoptosis in a series of cancer cell lines.

Effect of the combination of BLM and the different molecules on ROS generation

We have previously reported that BLM induced ROS production that is involved in its apoptotic effect (Brahim et al. 2007; Millet et al. 2002). In order to determine whether BLM in combination with the different agents tested enhances ROS production that may explain their sensitization effect, we then analyzed the intracellular generation of

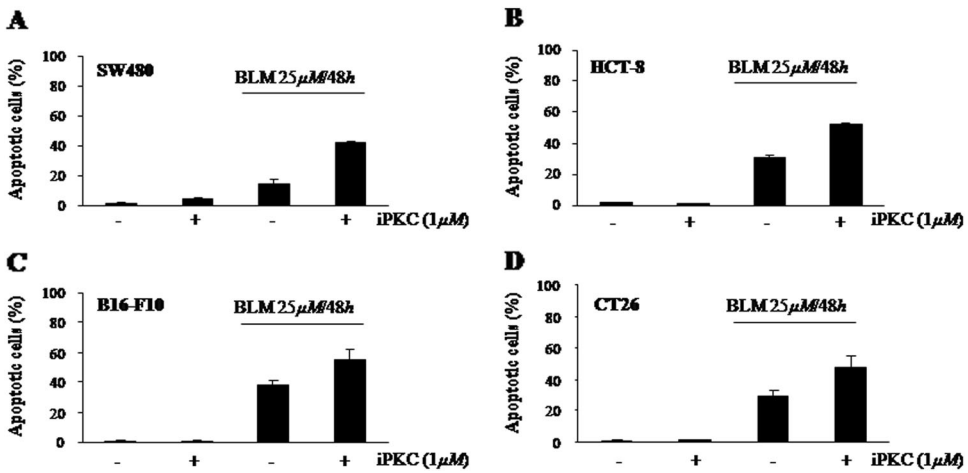

Fig. 2 Protein Kinase C inhibitor sensitizes various cancer cells to BLM-mediated apoptosis. SW480 (**a**), HCT-8 (**b**), B16-F10 (**c**) and CT26 (**d**) cells were treated either with 25 µM of BLM alone or in presence of 1 µM PKC inhibitor (iPKC) chelerythrine for 48 h. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye. In untreated cells, the percentage of apoptosis was always lower than 5%. Data represent mean ± SD (*bars*) of three independent experiments

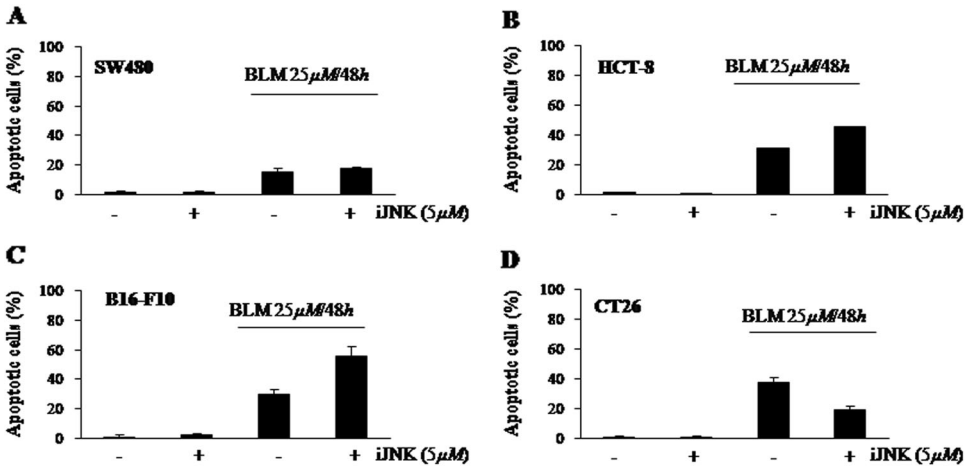

Fig. 3 JNK inhibitor sensitizes various cancer cells to BLM-mediated apoptosis. SW480 (**a**), HCT-8 (**b**), B16-F10 (**c**) and CT26 (**d**) cells were treated either with 25 μM of BLM alone or in presence of 5 μM JNK inhibitor (iJNK) SP600125 for 48 h. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye. Results are the mean ± SD (*bars*) of three independent experiments

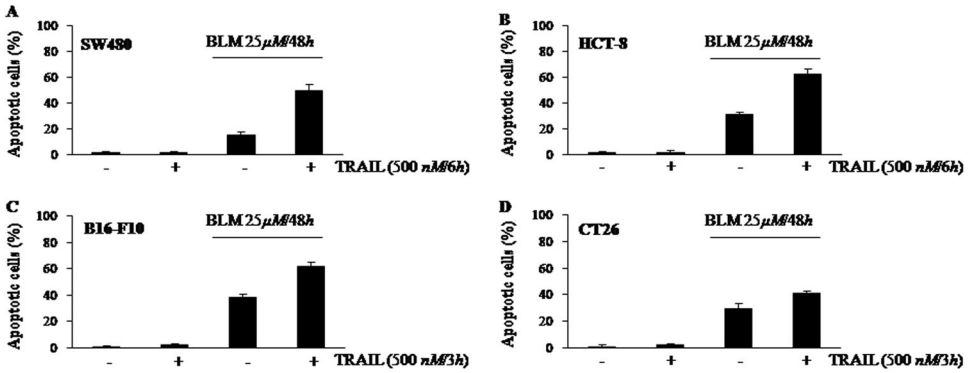

Fig. 4 TRAIL sensitizes various cancer cells to BLM-mediated apoptosis. Apoptosis of SW480, HCT-8, CT26 and B16-F10 cells were determined by Hoechst 33342 staining after a co-treatment of cells with 25 μM BLM for 48 h and 500 nM of recombinant soluble TRAIL for 6 h for SW480 and HCT-8 (**a**, **b**) and for 3 h for B16-F10 and CT26 (**c**, **d**). Results are the mean ± SD (*bars*) of three independent experiments

ROS by using dihydro-ethidium fluorescence, a probe that is more sensitive to superoxide anion than other ROS. As shown in Fig. 5, treatment of cells with 25 μM BLM clearly induced ROS production in three cell lines except for HCT8 in which little if not no ROS were produced. Such effect was differently modulated depending of the cell type and the agent. For instance, chelerythrine and TRAIL in SW480, HCT-8 and CT26, and SP600125 in HCT-8 and CT26 did not significantly modify the level of ROS produced by BLM. In contrast, these three latter molecules significantly enhanced BLM-triggered ROS production in B16-F10.

Collectively, these results suggest that the sensitization effect observed with the different agents is not correlated to their ability to raise ROS production induced by BLM in the different cell lines.

Involvement of ROS in apoptosis induced by BLM combined with the different agents

To confirm that ROS are not involved in cell death sensitization triggered by the combination of BLM and the different agents, we examined the effects of *N*-acetyl-cysteine (NAC), a broad ROS scavenger, on apoptosis induced in cancer cells treated by the different combinations. We found that treatment of cells with NAC caused a decrease of apoptosis induced by 25 μM BLM alone in SW480 and CT26 cells. This effect was not observed in HCT-8 and B16-F10 cells (Fig. 6). Conversely, NAC did not affect sensitization of SW480, HCT-8, and CT26 cells to apoptosis induced by BLM combined to chelerythrine, or TRAIL. However, NAC caused a significant decrease of apoptosis in CT26 cells treated by BLM and SP600125 (Fig. 6b). It is worthy to note that in melanoma cells apoptosis decrease by SP600125 was overcome by NAC (Fig. 6b). Collectively, these results raise the possibility that ROS may contribute to the synergistic effect of the different agents on the response to BLM in some but not in all cancer cells.

BLM is frequently used as a chemotherapeutic agent to treat various kinds of malignancy. However, the cytotoxic effects of BLM, related to an overproduction of ROS, can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis (Chaudhary et al. 2006; Grande et al. 1999; Gu et al. 2015; Tobwala et al. 2013). This limitation had pointed researchers to combinative therapeutic strategies, because dose-related toxicity has been one the major limiting factors in BLM-based therapies. To design a better combinative chemotherapeutic regimen there should be a better focus on

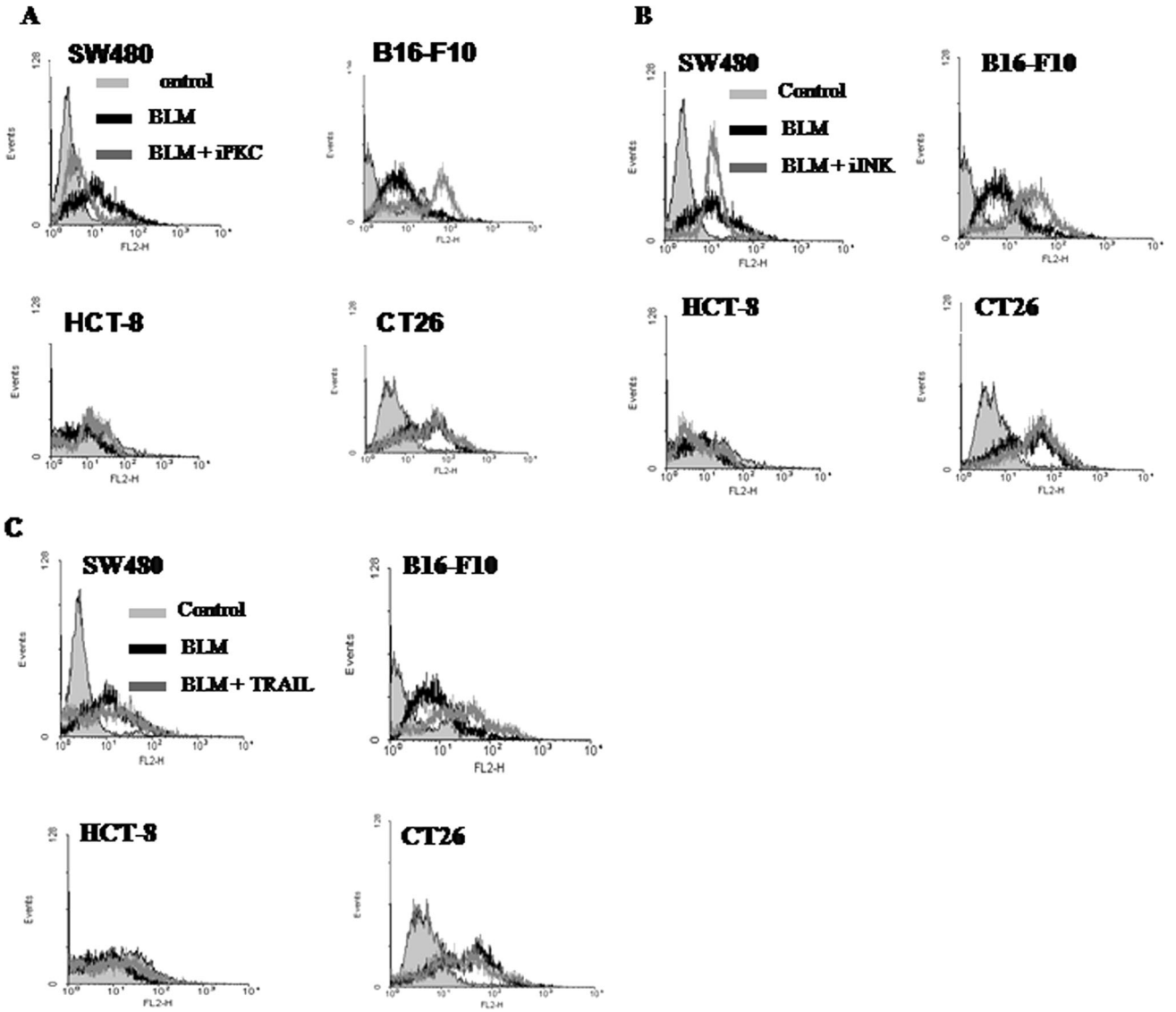

Fig. 5 SW480, HCT-8, CT26 and B16-F10 cancer cells were treated with 25 μM BLM alone or in the presence of 1 μM chelerythrine (iPKC) (**a**), or 5 μM of JNK inhibitor (iJNK) SP600125 (**b**) for 48 h. Cells were treated with 25 μM BLM for 48 h followed by 3 h-incubation with 500 nM of recombinant soluble TRAIL (**c**). ROS were measured by flow cytometry after treatment of cells with dihydroethidium (DHE). Results are representative of three independent experiments

cancer cell killing, we focused on a relative low dose of BLM in an associative treatment strategy. Our data demonstrated that treatment of cancer cells, representing different type of cancers, e.g., colorectal, and melanoma tumors, with low dose of BLM alone had moderate effect on apoptosis on these cells. In contrast, co-treatment with BLM and different apoptosis-regulators, including an apoptosis inducers (TRAIL), and two protein kinase inhibitors (iPKC and iJNK), results in a significant synergistic effect rather than a simple additive therapeutic effect. However, the degree of these synergisms varies in accordance with the type of cells and the drug nature. Indeed, whether JNK inhibitor sensitized BLM to apoptosis in two colon cancer cell lines (human HCT-8, and murin CT26 cells), it had not effect in the human colon cells SW480, but it significantly decreased BLM-triggering apoptosis in the murin melanoma B16-F10 cells. This latter result is in accordance with other data observed in leukemia and oral cancer cells (Brahim et al. 2007, 2008), suggesting that JNK inhibition is not suitable for an associative treatment strategy, at least with BLM in melanoma cancer. The unsuitable synergitic cytotoxic effect of genotoxic drug and JNK inhibitors which was also observed in melanoma cell lines (Selimovic et al. 2008). The two other molecules (iPKC, and TRAIL) displayed sensitization with BLM whatever the origin of the cells. The more interesting finding is that in B16-F10, the more aggressive and non immunogenic melanoma cancer cells (Fidler 1975), BLM rendered these cells

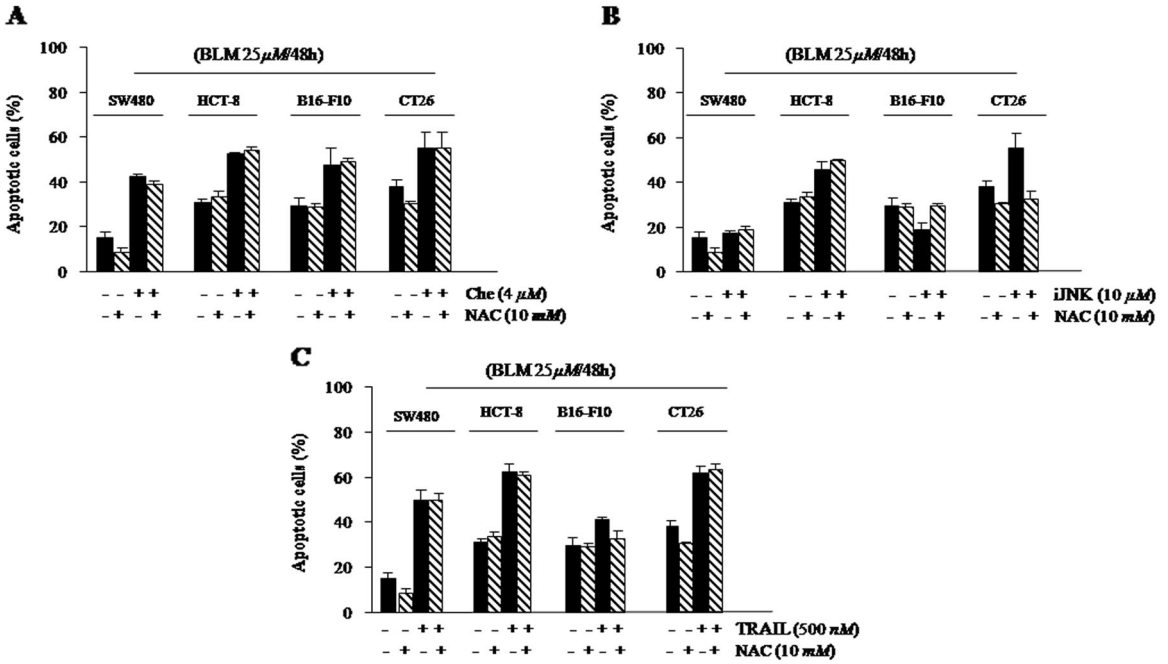

Fig. 6 Effect of NAC on apoptosis induced by BLM combined with the different agents. SW480, HCT-8, CT26 and B16-F10 cancer cells were treated with 25 μM BLM alone or in the presence of 1 μM chelerythrine (**a**), or 5 μM SP600125 (**b**) with or without 10 mM NAC for 48 h. Cells were treated with 25 μM BLM for 48 h followed by 3 h-incubation with 500 nM of recombinant soluble TRAIL (**c**) in the presence or not of 10 mM NAC. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye. Results are the mean ± SD (*bars*) of three independent experiments

more sensitive (up to 60% apoptotic cells) to a short stimulation with TRAIL (~3 h). Almost of cells are died after or over 6 h-treatment with TRAIL (data not shown), suggesting that BLM may represent a well sensitizer drug to TRAIL receptor agonists (Quast et al. 2013; Min et al. 2014), some of which are in clinical trials (Papenfuss et al. 2008). Although; the precise mechanism remains to be identified, our preliminary results indicate that some combinations displayed more or unchanged ROS production when compared to BLM or to molecules taken alone. Further, in two treatment conditions (Fig. 6b and c), scavenging of ROS significantly decreased apoptosis, suggesting that ROS could be involved in sensitization in certain cancer cells (Zhang et al. 2016; Provinciali et al. 2015), and could be not required for apoptosis in some others (Burgy et al. 2016; Park et al. 2015).

Conclusion

The combination of BLM with some compounds, including TRAIL agonists, or iPKC may represent new strategies to overcome chemotherapy resistance of tumors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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