Biochemical Pharmacology 79 (2010) 1589–1599

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Bax deficiency mediated drug resistance can be reversed by endoplasmic reticulum stress induced death signaling☆

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ARTICLE INFO

Article history:
Received 6 December 2009
Accepted 26 January 2010

Keywords:
Drug resistance
ER stress
Bax mutation
Apoptosis
Autophagy

ABSTRACT

Tumors often acquire drug resistance due to functional loss of pro apoptotic gene Bax, a critical and essential component of cell death rendering them insensitive to most anti-tumor agents. Compounds that can induce Bax independent apoptotic cell death are expected to overcome such drug resistance. We have employed a live cell based screening platform to identify potential compounds that can induce programmed cell death in Bax deficiency. Release of cytochrome C from mitochondria into the cytosol is a decisive initial event required for the caspase dependent cell death. We have engineered both wild type and Bax knock out colon cancer cells stably expressing cytochrome C with EGFP fusion protein to identify compounds that can trigger cytochrome C release in both cells with equal efficiency. In the fluorescent translocation assay, most of the drugs tested failed to induce cytochrome C release in Bax deficient cells validating the sensitivity of the assay. This study identified five lead compounds such as thapsigargin, tunicamycine, MG132, kaempferol and camptothecin that could induce cytochrome C release in both wild type and Bax deficient cells with equal potency. All the positive hits induced ER stress signaling as evidenced by up-regulation of Grp78. Studies with a Bak deficient cells indicate that Bak deficiency confers protection to cells from ER stress through autophagy. Further studies revealed that ER stress inducing agents are capable of triggering classical mitochondrial apoptotic cell death through the conformational activation of Bak, substantiating the potential of this pathway in designing drugs against Bax deficiency mediated drug resistance.

1. Introduction

Therapy resistant tumors often arise as a result of loss or mutational inactivation of essential genes involved in tumor suppression or apoptosis execution. This poses a major concern in the treatment of a variety of tumors since most known anti-tumor agents require essential components of classical death signaling pathways to trigger target cell apoptosis. Colon cancers are relatively resistant to most conventional anti-tumor drugs and this resistance is closely linked to the loss of apoptosis signaling [1–5]. One of the important multi-domain pro apoptotic Bcl2 family proteins essential for initiating apoptotic cell death is Bax and subsets of colon cancers are found to be associated with Bax mutation. Its conformational activation triggered by a still unknown mechanism during apoptosis contributes for cyt.C release from mitochondria, leading to caspase mediated cell death [6–8]. A large number of antitumor agents are known to induce conformational activation of Bax [9,10].

The multi-domain pro-apoptotic proteins Bax/Bak are essential and redundant regulators of a diverse intrinsic mitochondrial cell death pathway [11]. Bax/Bak double deficient murine embryonic fibroblasts (MEFs) are resistant to multiple apoptotic stimuli that increase outer mitochondrial membrane permeability, including staurosporine, ultraviolet radiation, growth factor deprivation and etoposide [12,13]. Functional inactivation of Bax may have multifaceted effects ranging from induction, progression and regression of tumors as well as the clonal evolution of drug resistant tumor cells leading to failure of treatment [9,12,14]. It has been found that frame shift mutations in the [(G) 8] tract of exon 3 of the *Bax* gene occurs as a result of mutation in some of the important mismatch repair proteins. This accounts for the therapy resistance in familial colorectal cancers [15,12,16,17].

The situation therefore highly demands for effective therapeutic formulations capable of bypassing drug failure of such tumors with Bax mutation in order to improve the sensitivity of treatment methodologies like radiation and chemotherapy. Since the release

☆ This work was supported by funding from Department of Biotechnology (IYBA Award), Department of Science and Technology, University Grant Commission, and International Foundation of Science (Sweden).

Abbreviations: BaxKO, Bax knock out; cyt.C-EGFP, Cytochrome C-enhanced green fluorescent protein; ER, Endoplasmic reticulum.

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doi:10.1016/j.bcp.2010.01.032

of cyt.C is blocked in Bax deficient cells upon drug treatment, screening of compounds capable of inducing cyt.C release in Bax deficient background appears to be the best strategy for identifying potent anti-tumor agents capable of inducing apoptotic cell death in the absence of Bax. To address this issue, cells stably expressing cyt.C-EGFP in wild type HCT116 cells and BaxKO cells were generated. Using this cell based assay, we have identified several lead molecules that could induce cyt.C release in the absence of Bax. Most of the positive hits identified appear to induce ER stress mediated death signaling indicating that this pathway can be exploited for addressing drug resistance in colon cancers with Bax mutation. We found that Bak is the potent target of these compounds to initiate apoptotic cell death, the absence of which accounts for autophagy mediated survival.

2. Materials and methods

2.1. Cell culture maintenance and treatment

Human colon cancer cell lines, HCT116 and BaxKO cells were obtained from Dr. Bert Vogelstein, John Hopkins School of Medicine, Baltimore. The cells were maintained in Mc Coys (Invitrogen, USA) medium containing 10% Fetal Bovine Serum and antibiotics. The breast cancer cell line SKBr3, colon cancer cell lines LoVo and SW480 were obtained from American type culture collection (ATCC, USA) and maintained in Dulbecco's Modified Eagle's medium containing 10% FBS and antibiotics in a humidified CO_2 chamber at 37 °C.

2.2. Reagents and expression vectors

Thapsigargin (TG), MG132 (MG), tunicamycine (Tuni), ionomycine (Iono), kaempferol (Kae), staurosporine (ST), 5-fluorouracil (5-FU), 5-hydroxy urea (5-HU), camptothecin (Cam), cisplatin (Cis), vinblastin (Vin), epigallocatechin gallate (EGCG), myricetin (Myr), resveratrol (Res), colchicine (Colch), nocodazole (Noco), quercetin (Que) and other antitumor agents were obtained from Sigma Chemicals, USA. Antibodies against Grp78 (sc-13968) and actin (sc-1616) were obtained from Santa Cruz Biotechnology, USA. Antibodies against caspase 8 (#551243), cyt.C (#556432) and caspase 9 (#556585) were obtained from BD Biosciences, USA. Antibodies against caspase 3 (#9668), cleaved caspase 9 (#9501) and LC3B (#2775) were purchased from Cell Signaling Technology, USA. Anti-bak (AM04) antibody was obtained from Calbiochem, USA. Anti-mouse and anti-rabbit secondary antibodies conjugated with horse radish peroxidase were obtained from Sigma.

The expression vectors Bak-EYFP and RNAi vector for Bak (pBS/U6 BakRNAi) were kindly provided by Dr. Stanley Korsmeyer and Dr. Chinnadurai respectively. Full length mouse cyt.C cloned in pEGFP-N1 vector was kindly provided by Dr. Douglas Green [18–20]. The caspase 3 specific FRET probe, YRec expressing EYFP—DEVD-DsRed was obtained from Dr. Takuo Suzuki, National Institute of Health Sciences, Tokyo [21]. The expression vector pEGFP-LC3 was kindly supplied by Dr. Tamotsu Yoshimori and Dr. Noboru Mizushima, Tokyo Medical and Dental University Tokyo [22].

2.3. Generation of stable cell lines for monitoring cyt.C release

The colon cancer cells HCT116 and BaxKO cells were transfected with cyt.C-EGFP as per standard protocol and were maintained in 800 μg/ml of G418 containing medium for one month. Among 100 clones selected and expanded, only the clone that expressed cyt.C with the correct mitochondrial targeting were further expanded and used for the current study. The correct mitochondrial localization was verified by staining the cells with Mitotracker Deep Red (Molecular Probes, USA).

2.4. Analysis of chromatin condensation

After treatment with different drugs, the cells were stained with Hoechst 33342 dye (1 μg/ml) and incubated for 10 min at 37 °C before imaging under UV filter using Epi-fluorescent Microscope (Nikon TE 2000E). Cells with condensed or fragmented nuclei were taken as the apoptotic population and counted against total number of cells in the field and plotted graphically with percentage of apoptotic cells against the treatments.

2.5. Imaging of cyt.C-EGFP

Cyt.C-EGFP expressing cells were grown on 96 well glass bottom plates (BD Biosciences, USA) for 24 h and treated with different drugs for indicated time periods. The cells were directly viewed under fluorescent microscope TE 2000E equipped with a CARV confocal attachment (Becton Dickinson) and automated excitation and emission filter wheel with 60× Plan Apo 1.4 NA objective. The filter configuration used for EGFP includes excitation filter of 470/30 and Emission filter of 520/40. The images were captured in non-confocal mode with an EMCCD camera Rolera Mgi (Q imaging) using IP Lab software (BD Biosciences, USA).

2.6. Live cell caspase 3 monitoring by FRET microscopy

SKBr3 cells were transfected with the expression vector for caspase 3 FRET probe by lipofectamine 2000 as per the manufacturer's instruction (Invitorgen, USA). The cells stably expressing FRET probe was selected in G418 selection medium. The cells expressing high FRET values were enriched by flow sorting based on its red vs green signal using FACSAria. For fluorescence detection of FRET by microscopy, cells were seeded on chambered cover glass (Nunc, Denmark) and after indicated treatment, images were collected using CARV II unit mounted on an Epi fluorescent microscope TE 2000E (Nikon). A single excitation of EYFP at 472/30 and dual emission of EYPF at 442/27 and DsRed at 624/40 (FRET channel) was collected using automated excitation and emission filter wheel controlled by IP Lab software (BD Biosciences, USA).

2.7. Immunofluorescence detection of cyt.C and LC3

Cells grown on chambered cover glass after indicated drug treatment were fixed with ice cold acetone–methanol (1:1) for 10 min and stained with primary antibody against cyt.C or LC3 after blocking with 2% BSA in TBST. Alexa fluor 488 or 556 conjugated secondary antibodies (Molecular Probes, USA) were used to visualize the signal and imaged after mounting with slowfade (Molecular Probes, USA). The cells were imaged using fluorescent microscope TE 2000E equipped with a CARV II confocal attachment using specific filter combinations. In separate experiments SKBr3 cells were transfected with pEGFP-LC3 expression vector and cells with moderate level of LC3 expression were sorted by FACSAria. The cells were seeded on coverslips and treated as indicated. Cells with autophagy like changes were scored based on GFP aggregates in the cytosol.

2.8. Analysis of conformational activation of Bak

The cells after indicated treatment were trypsinised and fixed with 3.7% para formaldehyde. The cells were permeabilized with 0.001% CHAPS buffer for 10 min and incubated with an antibody that recognizes N-terminal exposed conformationally active Bak (TC 100-Calbiochem, USA). FITC conjugated secondary antibody was used to develop the signal. Total fluorescence from 10,000 cells was collected by FACSAria.

2.9. Western Blotting

Cells after indicated treatments were scraped off and washed with ice cold PBS. The washed cell pellet was lysed in phospholysis buffer containing NP-40 and protease inhibitors and incubated in ice for 30 min. 50 μg protein samples was resolved electrophoretically on 10% or 12% SDS-polyacrylamide gel and electro transferred to a polyvinyl difluoride membrane (Amersham, USA) as per the wet transfer procedure (Bio-Rad Mini Protean II). The membrane after blocking in 5% skimmed milk powder in 1× TBST was incubated with primary antibody overnight in cold followed by secondary antibody for 1 h at room temperature. The signal was developed by enhanced chemi-luminescence assay (Amersham, USA) or by DAB.

2.10. Lysosomal integrity analysis by lysotracker red staining

SKBr3 cells were seeded on 12 well plates and treated with TG (1 μM), MG132 (1 μM), tuni (2 μM) or ST (1 μM) for 12 h. The trypsinised cells were stained with 50 nM of lysotracker red from Molecular Probes (L 7528) and incubated for 30 min at 37 °C in 5% CO_2. The red fluorescence from 10,000 cells was collected using FACS Aria.

3. Results

3.1. Development of BaxKO cyt.C-EGFP cells and screening of compounds

Bax deficiency culminates in the failure of cyt.C release in response to a variety of apoptosis inducing agents. To identify compounds that can induce programmed cell death in a Bax independent manner; we have generated cell lines for live cell monitoring of cyt.C release. Both wild type and BaxKO cells were transfected with cyt.C-EGFP expression vector and cells with properly targeted cyt.C were generated by colony selection followed by FACS sorting. The different clones expressing cyt.C-EGFP were separately expanded and verified for localization of cyt.C by co-staining with mitotracker red. Among 100 clones expanded, only a few clones expressed cyt.C with proper localization at mitochondria (Fig. 1). Best clones with correct targeting of cyt.C and functional competence for release upon drug treatment as that of native cyt.C were expanded and used for further experiments. In order to visualize the release of cyt.C-EGFP upon apoptosis, the cells were exposed to a known apoptosis inducer etoposide and the green fluorescence was visualized under fluorescence microscope. The mitochondria were stained using mitotracker deep red and nucleus

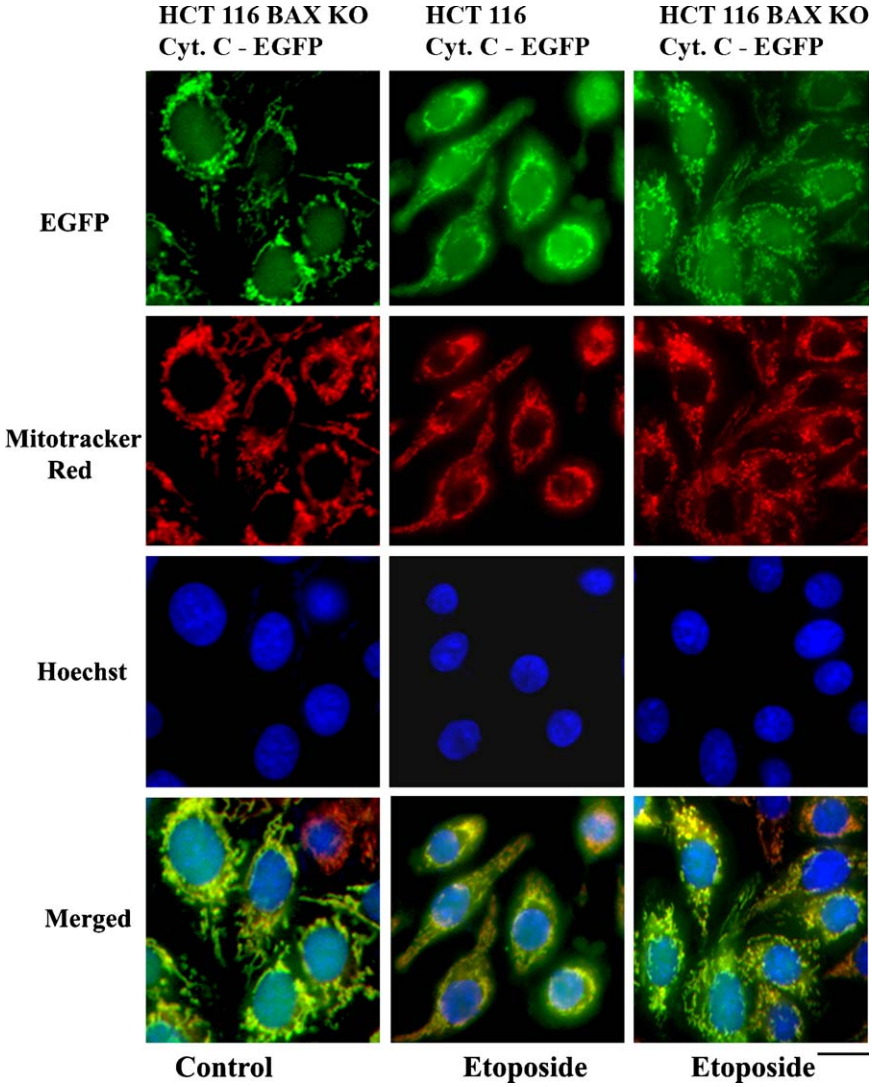

Fig. 1. Colon cancer cell HCT116 and BaxKO cell expressing cyt.C-EGFP were generated as described. The cells after exposure to 50 μM of etoposide for 12 h were stained with mitotracker red and hoechst dye and imaged under epi-fluorescent microscope. The green channel represents cyt.C, red mitochondria and blue nuclei (scale bar: 20 μm).

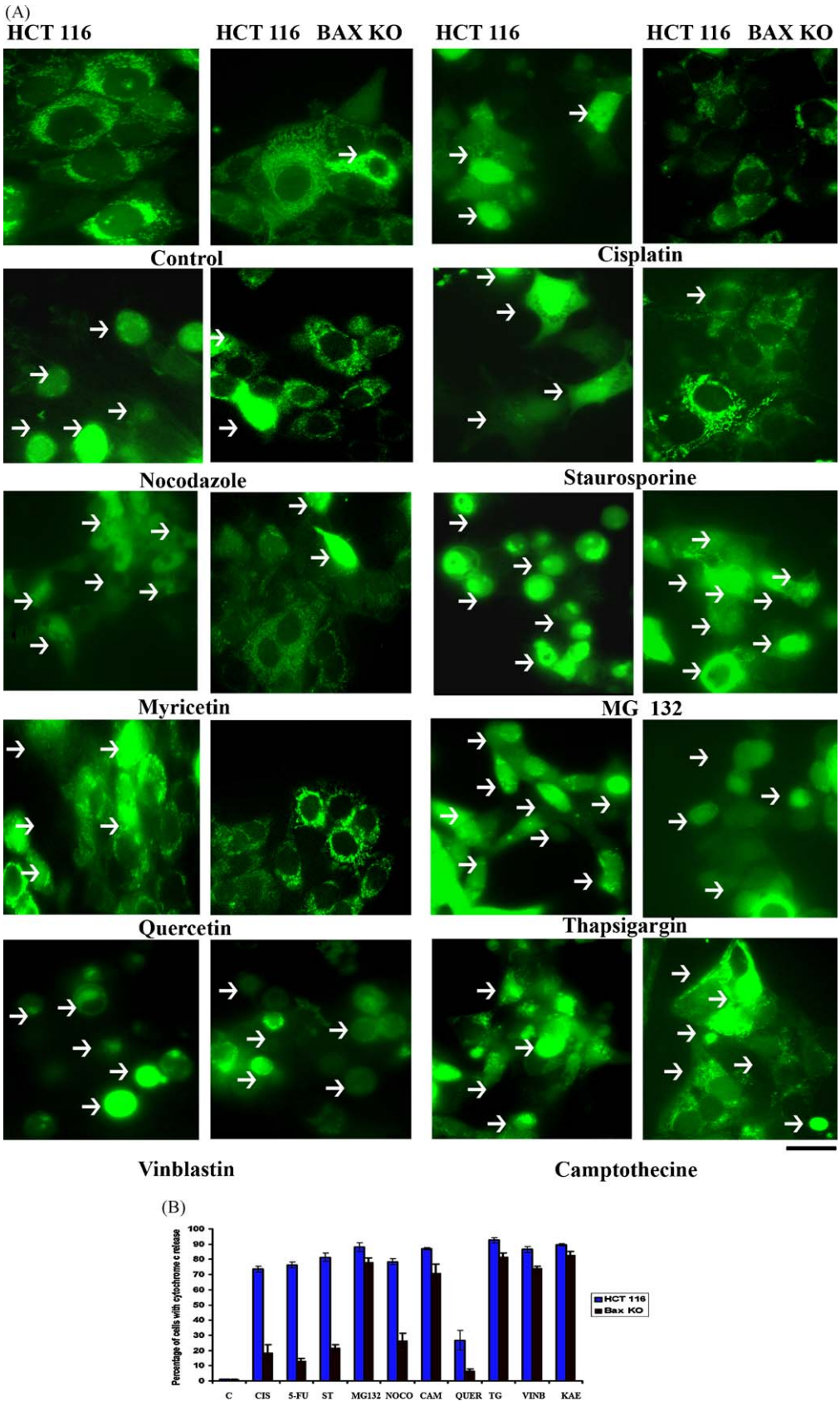

Fig. 2. (A) HCT116 and BaxKO cells expressing cyt.C-EGFP were exposed to different drugs for 12 h and imaged under fluorescent microscope. In the control cyt.C expressing cells showed a granular pattern indicating their presence at mitochondria. Drug treated cells shows more diffuse green fluorescence pattern indicating their release into cytosol and some cases with nuclear translocation indicated by arrows (scale bar: 20 μm). (B) HCT116 and BaxKO cyt.C-EGFP cells were exposed to different drugs for 12 h and imaged under fluorescent microscope. The cells with diffuse green cyt.C were counted among 200 cells per well by two investigators to calculate percentage positive cytochrome C released cells ($n = 4$).

by hoechst dye. Etoposide failed to induce cyt.C in BaxKO cells (Fig. 1) compared to wild type cells indicating the functional competence of Bax deficiency in providing resistance towards cyt.C release thereby validating the assay system.

3.2. Most anti-tumor agents failed to release cyt.C in BaxKO cells except thapsigargin, MG132, kaempferol and camptothecin

We have used anti-tumor agents with diverse mechanisms of action and several experimental apoptosis inducing compounds for the initial screening to identify Bax independent cyt.C releasing compounds. The important apoptosis inducing agents used for the current experiment and their known mechanism of action is given in supplementary Table 1. The IC-50 value for each compound was separately determined in HCT116 wild type cells by MTT assay. Based on these IC50 value, we have used different concentrations of the drugs for screening purpose. The cyt.C release was monitored under fluorescent microscope for 6, 12 and 24 h. Cells with diffuse cytoplasmic green fluorescence were scored as cyt.C released cells. Most of the apoptosis inducing agents induced the

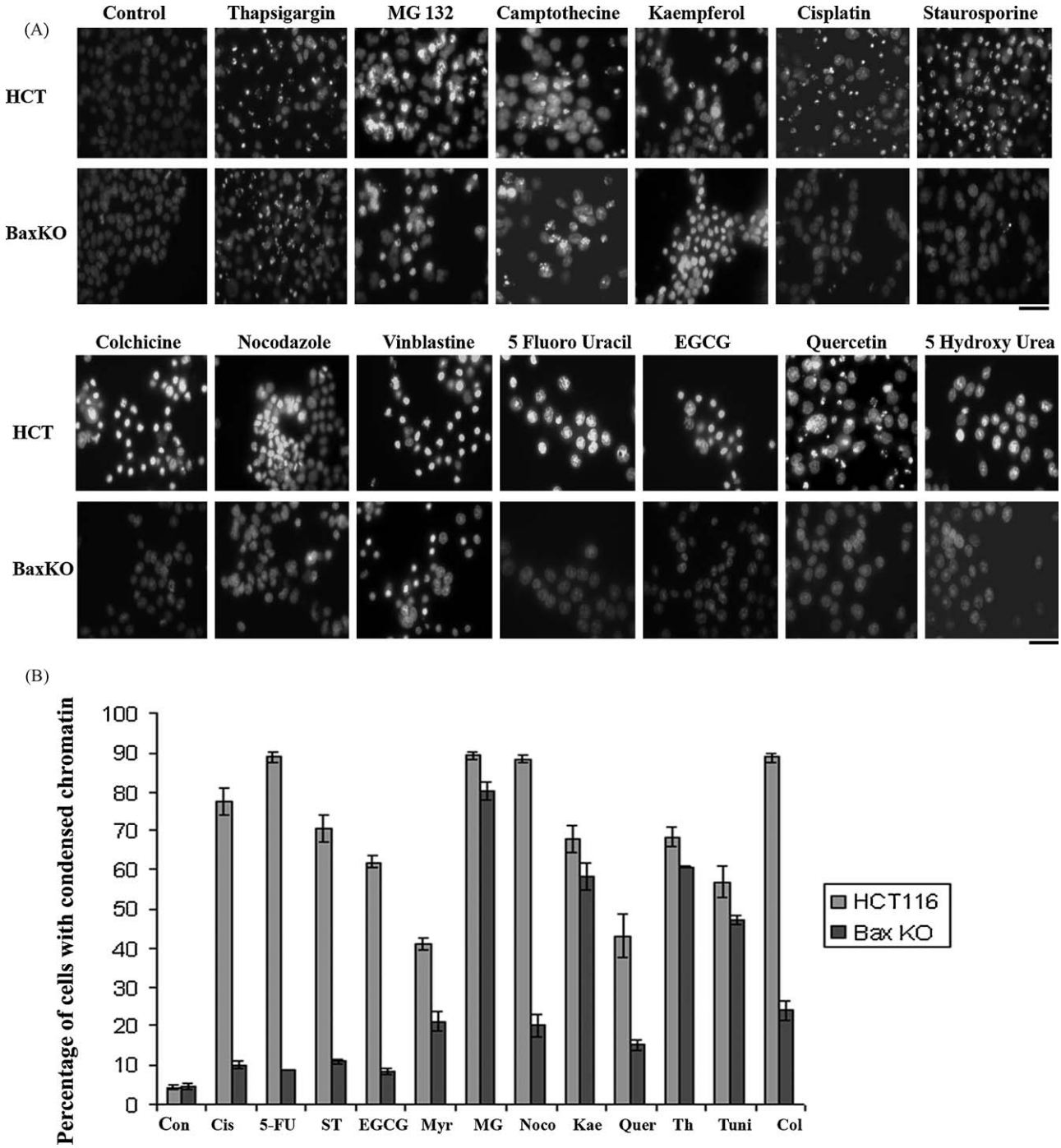

Fig. 3. (A) HCT116 and BaxKO cells were treated with thapsigargin (1 μM), kaempferol (100 μM), MG132 (1 μg/ml), vinblastine (1 μM), camptothecin (1 μM), cisplatin (25 μg/ml), staurosporine (1 μM), colchicine (5 μg/ml), nocodazole (100 nM), 5 fluoro uracil (10 μM), 5 hydroxy urea (200 μM), EGCG (100 μM), quercetin (100 μM), myricetin (100 μM) for 24 h and stained with Hoechst dye as described to visualize chromatin condensation under fluorescent microscope. The intensely stained nuclei represent cells with condensed chromatin (scale bar: 50 μm). (B) HCT116 and BaxKO cells were treated with indicated drugs for 24 h. The cells were stained with Hoechst dye to calculate the percentage cells with condensed chromatin. Results show mean values ± s.d. of 5 independent experiments.

release of cyt.C in wild type cells starting from 12 h at the respective concentrations indicated. Cyt.C release was markedly prevented in BaxKO cells except for TG, MG 132, Kae, Cam and Vin. These drugs even at a very low concentration induced cyt.C release in a Bax independent manner (Fig. 2A). For quantitative information, a total of 200 cells per well were analyzed for the pattern and percentage of cells with diffuse cyt.C for each drug. Percentage of cells with diffuse cyt.C for each drug is shown in Fig. 2B.

3.3. Most anti-tumor agents except above identified lead compounds induced Bax dependent cell death

Our experiments involving cyt.C expressing cells identified five potential compounds capable of inducing cyt.C release in both wild type and BaxKO cells with almost equal efficiency. Further, to understand whether the cyt.C release culminated in cell death, we have analyzed chromatin condensation, one of the important hallmarks of apoptosis, by staining with hoechst dye. Similar to the above results, we have observed that anti-tumor agents like 5-FU, Cis, ST, etc., induced chromatin condensation in wild type cells but not in BaxKO cells (Fig. 3A and B). However, Kae TG, Tuni, MG-132, Cam and Vin induced chromatin condensation both in wild type and BaxKO cells. Altogether, we have identified certain potential compounds that can induce programmed cell death independent of Bax.

3.4. The lead molecules induced ER stress

Despite diverse mechanisms of action, majority of the positive hits are reported to induce ER stress in a variety of cell lines. We have evaluated the potential of these compounds in triggering ER stress in wild type and BaxKO HCT116 cells by analyzing the up-regulation of ER stress specific marker Grp78. All the five positive hits significantly induced Grp78 in BaxKO cells compared to other anti-tumor agents (Fig. 4A–C). Most of the lead compounds upregulated Grp78 in a time dependent manner starting from 6 h (data not shown). A representative blot of cells treated with TG is shown in Fig. 4B. We have also analyzed caspase 9 and caspase 8 processing by Western Blot analysis after treatment with TG, Kae, MG, Cam and Vin in BaxKO cells. Consistent with the above data, TG, MG-132, and Cam significantly induced caspase 9 and caspase 8 processing in BaxKO cells (Fig. 4D).

3.5. The lead molecules induced Bak conformational activation

To understand whether the lead compounds could target Bak to induce cyt.C release, we have analyzed conformational activation of Bak using the antibody that specifically detects conformationally active Bak by FACS analysis. All the ER stress inducing agents significantly enhanced conformational activation of Bak but not 5-FU (Fig. 5A). The results indicate a possible role of Bak in inducing classical apoptosis signaling in BaxKO cells. All the lead compounds were capable of targeting Bak leading to its conformational activation. Further, we have silenced Bak in wild type and BaxKO cells and analyzed for cyt.C release by immunofluorescence technique. Cyt.C release was partially prevented in Bak silenced Bax proficient cells and it was completely prevented in Bak silenced Bax deficient cells treated with the lead compounds for 12 h, indicating the potential role of Bak in the regulation of cytochrome C release (data not shown). Another colon cancer cell line deficient for Bax protein, LoVo has been employed to test the efficacy of the lead compounds in the induction apoptosis and compared with Bax expressing colon cancer cell line SW480. The chromatin condensation data revealed that Bax deficiency rendered LoVo cells less responsive to cisplatin and 5 hydoxy urea, however all the lead compounds promoted chromatin condensations almost same as that of Bax proficient SW480 cells (Fig. 5B). Altogether the data support the ability of the lead compounds in triggering cell death even in the absence of Bax proteins.

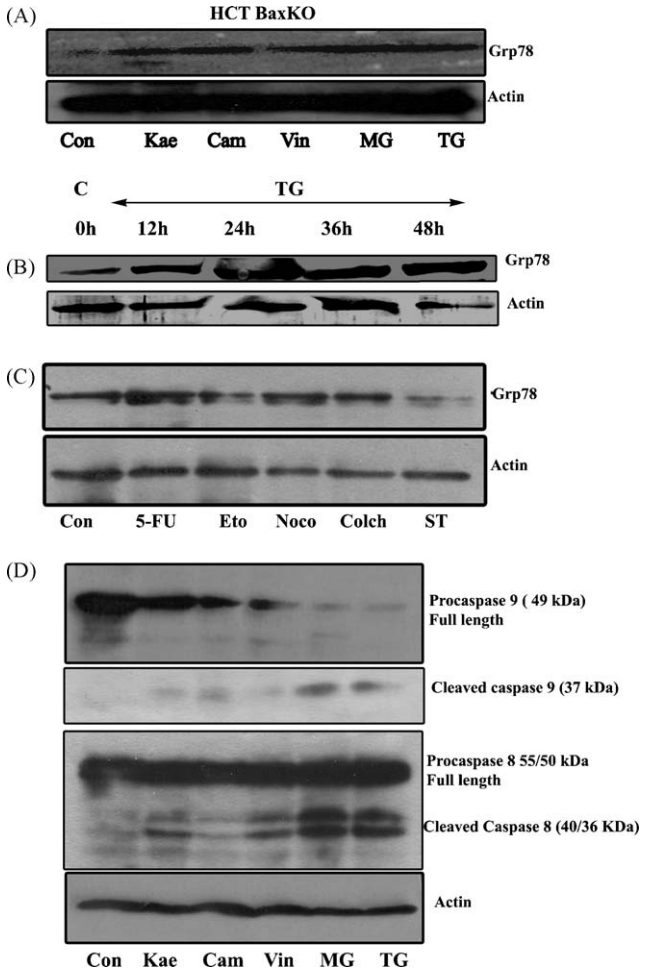

Fig. 4. (A) BaxKO cells were either untreated (control) or exposed to kaempferol (100 μM), camptothecin (1 μM), vinblastine (1 μM), MG-132 (1 μg/ml), thapsigargin (1 μM) for 24 h. Western Blot analysis was carried out for Grp78 up-regulation. β-Actin served as the loading control. (B) HCT116 cells were exposed to 1 μM of TG for 12, 24, 36 and 48 h. The whole cell extract was prepared as described and probed with Grp78 antibody. β-Actin served as loading control. (C) BaxKO cells were either untreated (control) or exposed to indicated drugs for 24 h. Western Blot analysis was carried out for Grp78 up-regulation. β-Actin served as the loading control. (D) BaxKO cells were exposed to indicated drugs for 24 h. The whole cell extract was prepared and developed with caspase 9, cleaved caspase 9 caspase 8, and β-actin antibodies.

3.6. ER stress inducers target Bak for apoptotic cell death

To understand if the dependency of Bak for ER stress induced cell death is a general phenomenon or a cell specific one, Bak deficient breast cancer cell line SKBr3 was employed. Consistent with the earlier results, TG failed to induce cell death in SKBr3 cells compared to ST. Even at 48 h, Bak deficient SKBr3 cells were resistant to cell death induced by ER stress, however massive cytoplasmic vacuolization was noticed in these cells indicating autophagy like changes which were not observed in ST treated cells (Fig. 6A). The activation of the major executioner caspase 3 was also detected only in ST treated cells but not in TG treated cells (Fig. 6B). To substantiate the caspase activation, we have employed a sensitive caspase 3 specific FRET expressing cell-based system for live cell monitoring of caspase activation by FRET microscopy. Both

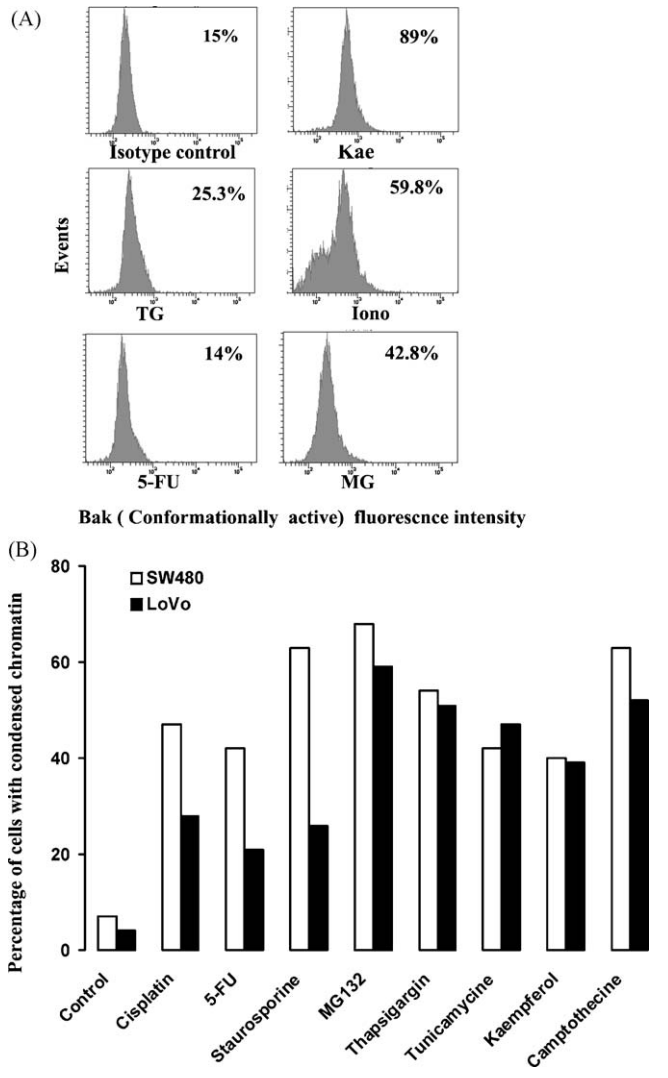

Fig. 5. (A) HCT116 cells were treated with different drugs for 24 h. The cells were trypsinised, fixed, permeabilized and stained with primary antibody against N-terminus of active Bak and with FITC conjugated secondary antibody. The fluorescent signal from 10,000 cells was collected by FACS. The percentage of cells with increase in immunoreactivity gated from the respective isotype control is also shown. (B) Colon cancer cell lines SW480 and LoVo were exposed to indicated drugs for 48 h and stained with hoechst dye to calculate the percentage cells with condensed chromatin as described. The average percentage of cells with condensed chromatin with each drug is shown ($n = 4$).

thapsigargin and kaempferol failed to induce significant FRET probe cleavage in SKBr3 cells even at 48 h as evidenced by increase in FRET signal (red) compared to staurosporine or 5 hydroxy urea that significantly cleaved FRET probe with loss of red signal dominating the green signal in merged image (Fig. 6C).

To substantiate whether the survival is correlated with autophagy like changes we have employed pEGFP-LC3 expressing cells to visualize the LC3 aggregation in live cells as an indication of autophagy [22]. Upon treatment with either thapsigargin or Kaempferol, 60–80% LC3 expressing cells showed cytoplasmic aggregation that was absent in staurosporine treated cells (Fig. 6D). Immunoblotting using LC3 antibody detected increased LC3 II 14 kDa band in SKBr3 cells treated with the ER stress inducing agents (Fig. 6E). These results substantiated that Bak deficiency promotes autophagy during ER stress. We have ectopically introduced Bak in SKBr3 cells with BAK-EYFP fusion protein and stained the cells using anti-LC3 antibody by immunofluorescence. In the transient transfection experiments, most of the EYFP expressing cells failed to show any LC3 granule formation and showed apoptotic morphology. On the other hand, 40% of the non-expressing cells showed LC3 aggregation (Fig. 6F). These observations clearly suggest that Bak deficiency associated cell survival during ER stress is mediated by autophagy. In the same manner, we have analyzed the cyt.C release and chromatin condensation after treatment with TG and ST in Bak-EYFP transient transfection experiments. cyt.C release and chromatin condensation was found restored in Bak-EYFP expressing cells, indicating the potential role of Bak in the regulation of mitochondrial cyt.C release and cell death (Supplementary Fig. 1).This observation was again supported by the data obtained from the analysis of lysosomal integrity by staining the cells with lysotracker red dye. FACS analysis of lysotracker red stained cells showed increased lysosomal stability in SKBr3 cells after ER stress compared to staurosporine treated cells further confirming the dominance of survival signaling in the absence of Bak during ER stress (Fig. 6G).

4. Discussion

Apoptosis plays a critical role in the regulation of developmental processes, tissue homeostasis and elimination of damaged cells. Mitochondria play a crucial role in apoptosis by releasing several apoptosis promoting proteins such as cyt.C, Smac/Diablo, and HtrA2/Omi into the cytosol [6,23]. After release, cyt.C binds to Apaf-1 to cause recruitment of caspase 9, which leads to the initiation of a caspase cascade that culminates in executioner caspase 3 activation and apoptotic cell death [9,24]. However, most solid tumors are resistant to apoptosis and evasion of apoptosis has been reported as a general mechanism of escaping drug induced toxicity [1,2,25,26]. Multiple modes of drug failures are reported among tumors as a result of defects in apoptosis signaling. In most cases the mitochondrial cyt.C release is prevented or reduced because of the over-expression of anti-apoptotic proteins like Bcl2 or mutational inactivation of Bax or Bak [7,9,10,27,28]. Bax and Bak are two important p53 regulated pro-apoptotic proteins that contribute for cell death induced by a variety of anti-tumor agents [9,10,4,29]. Mutation of Bax is frequently observed in MMR (mismatch repair) deficient tumors including human colon, gastric and endometrial cancers [9,12,30]. Inactivation of Bax is also reported as a major mechanisms of drug resistance in haematological malignancies [29,31]. Similarly Bak is mutated in a subset of breast tumors as a method of evasion from apoptotic cell death [27,32]. Studies in mice substantiated that complete cyt.C release is blocked in double knock outs of Bax and Bak [10]. Studies involving mice knocked out for Bax and Bak indicated that both these proteins are not mutually redundant for cell death [33].

We reasoned that Bax deficiency mediated drug resistance can be successfully attacked by agents that can release cyt.C from mitochondria by alternate pathway. In order to identify such compounds, we have employed BaxKO cyt.C-EGFP cells to search for cyt.C releasing compounds. Initially we chose immunofluorescence based detection of cyt.C release after drug treatment as a method to identify lead molecules. However, this method failed to provide any significant results primarily due to loss of apoptotic cells during fixation procedure and the remaining surviving cells often yielded confounding results. This prompted us to develop a cell based assay to detect cyt.C release by expressing Cyt.C-EGFP that enabled us to visualize the release of cyt.C in a systematic and kinetic mode. Several studies including ours substantiated that the fusion protein behaves just like that of native cyt.C [18–20,34].

5-Fluorouracil, the most frequently used drug for treating colorectal cancers triggers Bax dependent cell death as evident from cyt.C release and chromatin condensation consistent with the earlier reports [35]. Other widely used anti-tumor agents like

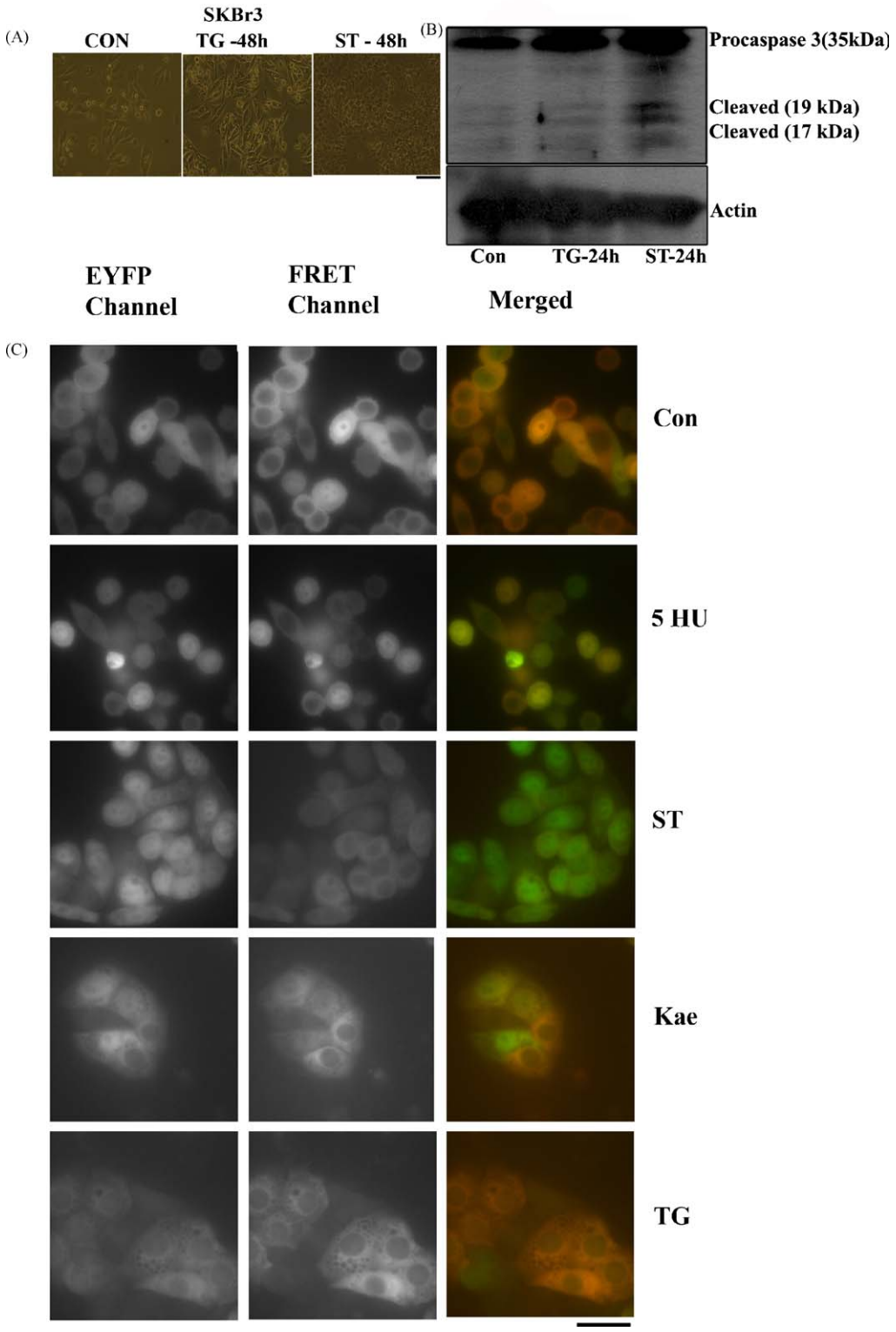

Fig. 6. (A) Bak deficient breast cancer cell line SKBr3 was treated with 1 μM of TG and ST for 48 h as described. The cytoplasmic vacuolization observed in TG treated cells are shown with bright field images (scale bar: 50 μm). (B) The whole cell extract prepared after indicated treatment was used for detection of caspase 3 by Western Blot. β-Actin served as loading control. (C) SKBr3 cells were transfected with caspase 3 FRET probe as described. Cells stably expressing FRET probe were treated with 5 hydroxy urea (200 μM), staurosporine (1 μM), kaempferol (100 μM), thapsigargin (1 μM) for 24 h before FRET imaging. The EYFP channel, FRET channel and merged images are shown. The change from red to green color indicates loss of FRET upon FRET probe cleavage (scale bar: 20 μm). (D) SKBr3 cells expressing EGFP-LC3 were treated with thapsigargine (1 μM), kaempferol (100 μM) and staurosporine (1 μM) for 24 h and visualized under GFP filter to see the LC3 aggregation. (E) SKBr3 cells were treated with tunicamycine (2 μM), thapsigargin (1 μM), and kaempferol (100 μM) for 24 h. The whole cell extract prepared was blotted for LC3B as described. The enhanced signal of 14 kDa LC3-II with loss of LC3-1 band indicates autophagy like changes. (F). The SKBr3 cells were transiently transfected with Bak-pEYFP and treated with thapsigargin or staurosporine. Fixed and permeabilized samples were immuno-stained with LC3 antibody and Alexa 546 conjugated secondary antibody. Imaging was carried out as described using specific filters for EYFP and Alexa Fluor 546. The percentage of cells with red LC3 aggregation were counted among a total of 200 EYFP expressing cells to calculate percentage positivity ($n = 4$). (G) SKBr3 cells grown on 12 well plates were treated with MG-132 (1 μg/ml), staurosporine (1 μM), and thapsigargin (1 μM), tunicamycine (2 μM) respectively for 12 h. The cells were stained with 50 nM of lysotracker red for 30 min at 37 °C. The cells were trypsinised and fluorescence from 20,000 cells was analyzed by FACS. Percentage of cells with increase in lysotracker red fluorescence intensity is indicated.

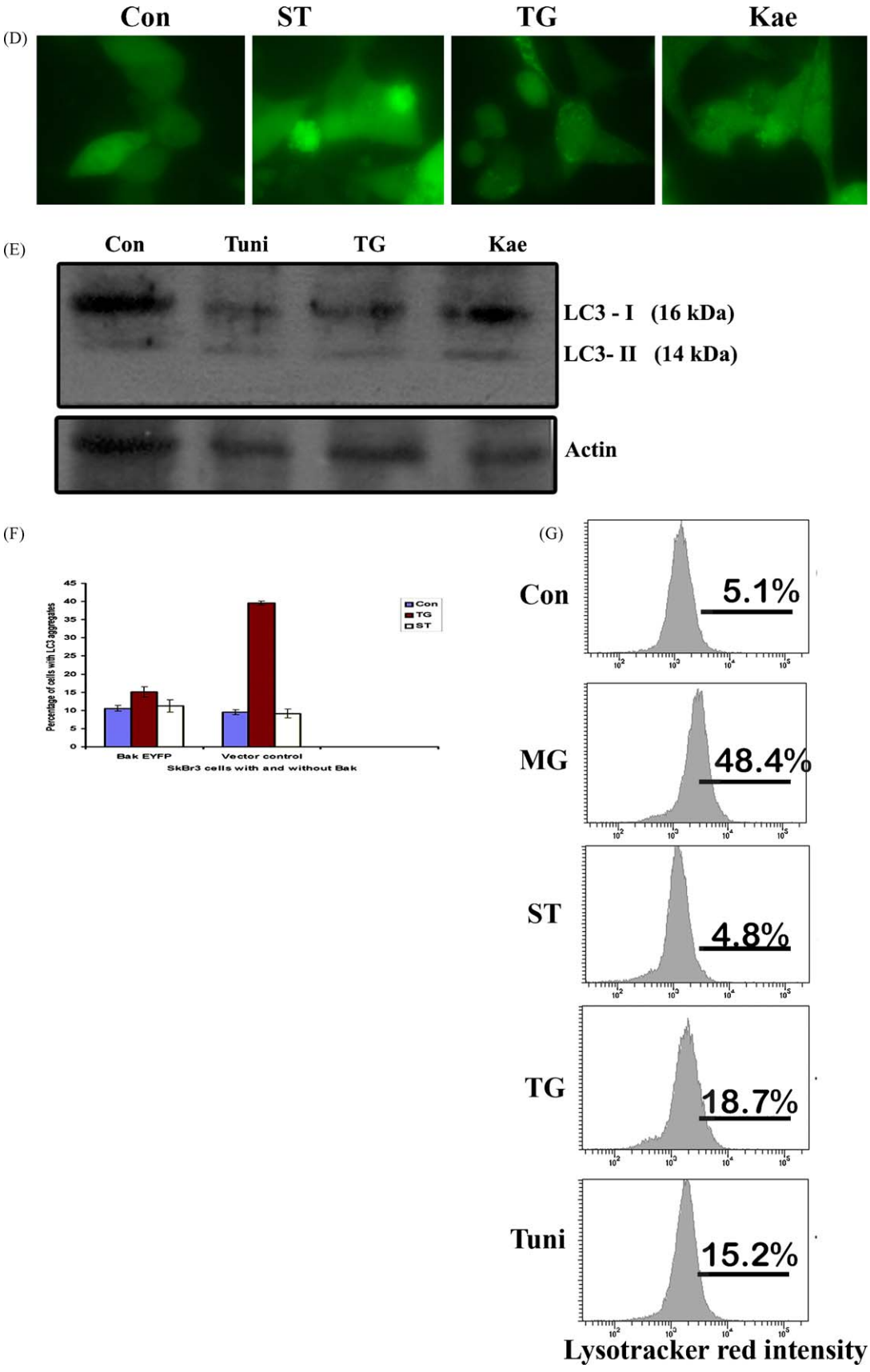

Fig. 6. (*Continued*).

cisplatin, staurosporine, and the microtubule destabilizing agents like colchicine, nocodazole, etc., also require Bax protein and failed to induce Cyt.C release in Bax deficient cells.

We have identified TG, Cam, Tuni, Kae and MG-132 as few promising compounds that could induce cyt.C release in both wild type and BaxKO cells with equal efficiency both at 12 and 24 h. Vin also induced a moderate level of cyt.C release in both HCT116 wild type and Bax KO cells at 24 h. Interestingly the lead compounds identified induced ER stress signaling in the target cells as evidenced by up-regulation of Grp78. Previous studies have indicated that TG, MG132 and Tuni are capable of inducing ER stress despite their diverse mechanisms of action [36,37]. The present study also indicated that along with these compounds the other lead compound Kae, Cam and Vin also induced moderate up-regulation of Grp78 indicating that ER stress inducers in general are capable of cyt.C release independent of Bax.

Although Bax and Bak share structural homology and functional equivalence, substantial differences in their regulation would be expected from their distinct localization in healthy cells [10]. Unlike Bax which is largely cytosolic, Bak resides in complexes on the outer membrane of mitochondria and endoplasmic reticulum of healthy cells [38,39]. On receipt of cytotoxic signals, both Bax and Bak undergo conformational activation and hetero-oligomerization leading to mitochondrial membrane permeabilization. Our studies further substantiated that the lead molecules identified, in general, target Bak and induce its conformational activation contributing to Bax independent cyt.C release. This was further substantiated in a Bak deficient cell, SKBr3 that failed to release cyt.C release upon ER stress induction until Bak is re-introduced. Bak introduction sensitized SkBr3 cells to cyt.C release and early apoptotic changes like externalization of phosphatidyl serine by annexin-V staining (data not shown).

Interestingly, ER stress induction in the absence of Bak initiated evident autophagy-like changes such as vacuolization and LC3 aggregation in SKBr3 cells, leading to cell survival even at 48 h duration of TG treatment. This result suggested that the survival response of SKBr3 cells under severe ER stress condition is brought about by autophagy in the absence of Bak. On the other hand, these autophagic changes were absent upon ST treatment that specifically targets Bax and cyt.C dependent cell death at early time points. This observation reinstated our hypothesis that Bak is essential for ER stress mediated cell death, the absence of which would lead to autophagy mediated cell survival. Ectopic expression of Bak-EYFP in SKBr3 cells showed much lesser LC3 aggregation and vacuolization along with more release of cyt.C and greater sensitivity to apoptosis upon TG treatment. Functional significance of Bak was further substantiated by RNAi silencing of Bak in HCT116 wild type and BaxKO indicating that the dependency for Bak for ER stress induced apoptosis is not tissue specific and is more or less a general mechanism.

Since all of the identified lead compounds induced ER stress signaling, it appears that Bak is primarily targeted by these agents rather than Bax. This is consistent with close association of Bak at ER membranes and earlier reports of conformational activation of Bak during ER stress. The recent findings of abundance of Bak, rather than Bax in intestinal epithelial cells and its role in colon epithelial cell development and differentiation, further supported the importance of this protein in designing drugs against Bax deficient tumors [40,15]. In contrast to the frequent early mutational inactivation of Bax, Bak is rarely mutated in colon cancers that too in only late stages in case of gastrointestinal cancers [9,12,14,30,40]. However, since we have observed an enhanced survival response in Bak deficient cells during ER stress, the expression status of this protein also should be considered during therapeutic intervention. Overall our studies highlight the importance of a general signaling pathway of ER stress response for effective drug development to address clinical drug resistance encountered in solid tumors. Most of the ER stress inducing agents like TG and tunicamycin are highly toxic and its clinical use is questionable. However, proteasomal inhibitors in general are already approved for clinical use and natural compounds like kaempferol and camptothecin offer great potential to be evaluated in preclinical studies to sensitize drug resistant tumors to therapy. Our studies substantiate that ER stress inducing signaling in general can be exploited for drug development against Bax deficient tumors and drug resistance.

Acknowledgements

The authors would like to thank Director, RGCB and the members of the Apoptosis and Cell signaling lab for their continued support. Assistance from Indu Ramachandran, Rajesh Kumar K. and Sudha B. Nair for flow cytometry is also acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.01.032.

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