Choline kinase inhibition induces exacerbated endoplasmic reticulum stress and triggers apoptosis via CHOP in cancer cells

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Endoplasmic reticulum (ER) is a central organelle in eukaryotic cells that regulates protein synthesis and maturation. Perturbation of ER functions leads to ER stress, which has been previously associated with a broad variety of diseases. ER stress is generally regarded as compensatory, but prolonged ER stress has been involved in apoptosis induced by several cytotoxic agents. Choline kinase α (ChoKα), the first enzyme in the Kennedy pathway, is responsible for the generation of phosphorylcholine (PCho) that ultimately renders phosphatidylcholine. ChoKα overexpression and high PCho levels have been detected in several cancer types. Inhibition of ChoKα has demonstrated antiproliferative and antitumor properties; however, the mechanisms underlying these activities remain poorly understood. Here, we demonstrate that ChoKα inhibitors (ChoKIs), MN58b and RSM932A, induce cell death in cancer cells (T47D, MCF7, MDA-MB231, SW620 and H460), through the prolonged activation of ER stress response. Evidence of ChoKIs-induced ER stress includes enhanced production of glucose-regulated protein, 78 kDa (GRP78), protein disulfide isomerase, IRE1α, CHOP, CCAAT/enhancer-binding protein beta (CEBPβ) and TRB3. Although partial reduction of ChoKα levels by small interfering RNA was not sufficient to increase the production of ER stress proteins, silencing of ChoKα levels also show a decrease in CHOP overproduction induced by ChoKIs, which suggests that ER stress induction is due to a change in ChoKα protein folding after binding to ChoKIs. Silencing of CHOP expression leads to a reduction in C/EBPβ, ATF3 and GRP78 protein levels and abrogates apoptosis in tumor cells after treatment with ChoKIs, suggesting that CHOP maintains ER stress responses and triggers the pro-apoptotic signal. Consistent with the differential effect of ChoKIs in cancer and primary cells previously described, ChoKIs only promoted a transient and moderated ER stress response in the non-tumorogenic cells MCF10A. In conclusion, pharmacological inhibition of ChoKα induces cancer cell death through a mechanism that involves the activation of exaggerated and persistent ER stress supported by CHOP overproduction.

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Phosphatidylcholine (PC) is a major structural component in eukaryotic membranes and a reservoir of the lipid second messengers diacylglycerol and phosphatidic acid. Choline kinase alpha (ChoKα) is the first enzyme of PC biosynthesis. Transformation of cells with the Ras oncogene (found in about 30% of all human tumors) increases both ChoKα activity and intracellular levels of phosphorylcholine (PCho), as also reported in several human tumors including lung, breast, colon, ovary and prostate. ChoKα is a critical requirement for breast tumor progression and its overexpression is considered as prognostic factor in lung cancer, and is associated with increased mortality in hepatocellular carcinoma. Specific ChoKα inhibitors (ChoKIs) or small interfering RNA (siRNA) inhibition have been proposed as a novel broad-spectrum antitumor strategy with proven antiproliferative activity against oncogene-transformed cells and human cancer cells. MN58b (1,4-[4-4'-Bis-([4-(dimethylamine) pyridinium-1-yl] methyl) diphenyl] butane dibromide) has demonstrated potent antiproliferative and antitumoral activity in vivo. MN58b has been used as a lead molecule for a first generation of compounds, synthesized to improve the tolerability of ChoKIs in vivo. RSM932A (also named TCD-717) has been selected among several molecules as it provided the best results in vitro and in vivo and is in the Phase I clinical trial for the treatment of solid tumors (http://clinicaltrials.gov/ct2/show/NCT01215864).

We have previously demonstrated that MN58b shows specific cytotoxic effects mediated by an increase of
ceramides in hematopoietic cancer cell lines, whereas primary cells suffer a reversible arrest in G1 being able to recover and proliferate once the drug is removed.\textsuperscript{23–25} However, the precise mechanism by which this selective effect is achieved is not fully understood.

Endoplasmic reticulum (ER) is a central organelle engaged in lipid synthesis, protein folding and maturation. Cellular perturbations, including hypoxia, failure of protein synthesis, folding, transport or degradation, Ca\textsuperscript{2+} overload and PC depletion can disturb the ER function resulting in ER stress.\textsuperscript{27–32} ER stress-induced apoptosis is becoming increasingly recognized as an important pathogenic factor in a vast number of diseases, including neurodegenerative diseases, diabetes, atherosclerosis and renal disease, and has been recently associated with the mechanism of action of antitumor agents such as vorinostat, sorafenib and bortezomb.\textsuperscript{33–38} ER disturbance triggers several specific signaling pathways, including ER-associated protein degradation and unfolded protein response (UPR).\textsuperscript{31,38,40} The UPR involves the activation of inositol-requiring protein1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6), which selectively suppress protein synthesis, promote the translation of specific proteins and regulate a wide variety of UPR target genes expression, including several ER resident chaperones as glucose-regulated protein 78 kDa (GRP78) and protein disulfide isomerase (PDI) or pro-apoptotic inducers as CHOP (also called GADD153).\textsuperscript{31,39,40}

CHOP is the major pro-apoptotic transcription factor induced by ER stress.\textsuperscript{39–42} Activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein beta (C/EBPβ) and CHOP complexes induce the transcription of several target genes that lead to ER stress resolution or apoptosis. One of these genes is TRB3, a novel target of CHOP/ATF4 and CHOP complexes induce the transcription of several target genes expression, including several ER resident chaperones as glucose-regulated protein 78 kDa (GRP78) and protein disulfide isomerase (PDI) or pro-apoptotic inducers as CHOP (also called GADD153).\textsuperscript{31,39,40}

We report that inhibition of PC synthesis by interfering with choline kinase activity triggers an exacerbated and persistent ER stress response that promotes cell death in breast, non-small-cell lung cancer (NSCLC) and colon-derived tumor cells. We identify CHOP as a key mediator in the cytotoxic effect induced by ChoK\textsubscript{z} inhibition. By contrast, non-tumorigenic cells exhibit a transient and attenuated ER stress response that contributes to survival due to overproduction of ATF4. Our results support that ChoK\textsubscript{z} inhibition is a bonafide target for developing highly specific novel antitumor cancer drugs.

**Results**

ChoK\textsubscript{z}s arrest cell cycle in non-tumorigenic cells but induce apoptosis in tumor cells. ChoK\textsubscript{z} inhibition by MN58b has been previously shown to selectively induce cell death in oncogene-transformed NIH3T3 cells and in Jurkat tumor cells, but is cytostatic in non-tumorigenic and primary cells.\textsuperscript{22,23} RSM932A has similar potential antitumor effects in several cell lines being more potent than MN58b in inhibiting ChoK\textsubscript{z} activity and intracellular PCho levels (data not shown).

Different epithelial mammary cell lines, including the tumor-derived cell lines T47D, MDA-MB231 and MCF7, and the immortalized non-tumorigenic mammary cell line MCF10A, were exposed to MN58b or RSM932A at 15 \( \mu \text{M} \) (around 5xIC\textsubscript{50}), for 24 or 48 h. Consistent with the previous published data in primary cells, ChoK\textsubscript{z} inhibition slowed down MCF10A cell growth suggesting the induction of cell cycle arrest, but reduction in cellular viability was not observed (Figure 1a). In contrast, cellular viability significantly decreased in all tumor cell lines studied (Figure 1a). Cell cycle modulation by ChoK\textsubscript{z}s was further evaluated by propidium iodide (PI) flow cytometry analysis. ChoK\textsubscript{z}s induced G1 phase cell cycle arrest in MCF10A cells but induced cell death in T47D (Figure 1b), MDA-MB231 and MCF7 tumor cell lines (data not shown). Furthermore, DAPI staining revealed small and bright nuclei and ‘apoptotic bodies’ in T47D cells (Supplementary Figure 1A), conversely, MCF10A nuclei structure was not modified after treatment.

Normal derived colon mucosa cell line NCM460,\textsuperscript{47} the colorectal cancer cell line SW620 and the lung tumor-derived cell line H460 were also analyzed to verify that the differential effects observed were not cell type specific. As in mammary cell lines and consistent with previous data, colorectal and lung cancer cells were triggered to apoptosis, whereas normal colon cells were arrested at G0/G1 after ChoK\textsubscript{z} inhibition with either RSM932A or MN58b (Figure 1c).

A marked reduction in cyclin D1, phosphorylated and total retinoblastoma protein (RB) and E2F1\textsubscript{z} was observed in tumor cells (Supplementary Figure 1B), suggesting a defec-tive regulation in the G0/G1 to S phase checkpoint. In non-tumorigenic cells, a significant reduction in cyclin D1 and phosphorylated RB (pRB) was also observed; however, E2F1\textsubscript{z} and RB levels were only slightly modified, indicating that G0/G1 arrest was due to the RB–E2F1\textsubscript{z} complex (Supplementary Figure 1B). Tumor cells showed pro-caspase 3 and PARP degradation after 24 and 48 h of treatment, suggesting that these proteins are not the main trigger mechanism for the apoptotic program (Supplementary Figures 1B and C).

ChoK\textsubscript{z}s induce exaggerated and persistent production of ER stress-related proteins in tumor cells, but promote moderated ER stress response in non-tumorigenic cells. ER stress response is triggered by different cytotoxic agents or intracellular toxic insults.\textsuperscript{39} Increased expression of ER stress-related genes was observed as early as 6 h after ChoK\textsubscript{z}s treatment, remaining elevated until 48 h, in T47D, MDA-MB231 and MCF7 tumor cell lines (Supplementary Figures 2 and 3). These genes were also upregulated between 9 and 48 h in non-tumorigenic MCF10A cells (Supplementary Figure 2). However, CHOP and C/EBPβ expression levels were higher in tumor cells than in the non-tumorigenic cells MCF10A (Supplementary Figures 2A and B), whereas ATF4 and TRB3 overexpression was markedly higher in MCF10A (Supplementary Figures 2C and D).
To further evaluate the effects of ChoKα inhibition on the ER stress signaling pathway, protein levels were analyzed. CHOP protein levels were significantly increased in T47D cells as early as 9 h compared with non-tumorigenic cells (Figure 2a), with a maximum at 24 h, and remained elevated after 48 h. Similar data were observed for C/EBPb (Figure 2b), GRP78 (Figure 2c), IRE1α (Figure 2d) and TRB3 (Figure 2f), and confirmed in MDA-MB231 tumor-derived cell line (Figure 2g). In T47D tumor cells, CHOP and C/EBPb were located in the nuclei of the majority of cells (Figure 3a). Conversely, although little effect was observed for all these proteins in the non-tumorigenic MCF10A cells (Figure 2a), ATF4 protein levels were significantly increased (Figure 2e), mainly in the nuclei (Figure 3b), suggesting that this transcription factor can have an important role as a pro-survival factor in non-tumorigenic cells.

PDI immunostaining showed a striking dilation in the ER of ChoKIs-treated cancer cells, a hallmark of the ER stress response (Figure 3c). PDI and CHOP proteins overproduction showed a similar effect that those observed in breast cancer cells (Supplementary Figure 4).

Figure 1  Choline kinase α inhibitors induce cell death in tumor cells and cause cell cycle arrest in non-tumorigenic cells. (a) MTT proliferation assay was performed in MCF10A non-tumorigenic cells, T47D, MCF7, MDA-MB231 breast cancer cell lines. Figures show data as mean ± S.E.M. of five independent experiments each one performed in quadruplicated. *P < 0.05 versus control cells after addition of ChoKIs. (b) Graph bars show DNA content analysis by PI, and flow cytometry data shown as mean ± S.E.M. of three independent experiments each one performed by duplicated. *P < 0.05 versus control cells. (c) Bars show DNA content analysis by flow cytometry data shown as mean ± S.E.M. of three independent experiments each one performed by duplicated. *P < 0.05 versus control cells.
CHOP overproduction induced by ChoKIs is attenuated when ChoKα protein levels are silenced. ChoKα siRNA assays were performed in order to confirm that the ER stress response induced by ChoKIs is due to their specific interaction with ChoKα. Although pharmacological inhibition of ChoKα induced a drastic alteration of CHOP, C/EBPβ and TRB3 (Figure 2), the partial ChoKα silencing achieved under these conditions failed to trigger ER stress in T47D (Figure 4a) and MDA-MB231 cells (Figure 4b). When cells were treated with RSM932A or MN58b for an additional 24 h after transfection with ChoKα siRNA for 48 h, the lower the levels of ChoKα, the more attenuated was the induction of CHOP by ChoKIs (Figure 4c and Supplementary Figure 5A), indicating that ER stress response induced by ChoKIs is triggered only in the presence of ChoKα. To support this view, in vitro binding experiments of ChoKα with its inhibitor RSM932A showed that this interaction induces a conformational change that can be followed by its sensitivity to trypsin digestion that could trigger ER stress responses and CHOP-dependent apoptosis (Supplementary Figure 5B).
Figure 3  (a) CHOP and C/EBPβ show nuclear location in T47D and MCF10A tumor cells after treatment with ChoKIs. Figure shows representative images of CHOP and C/EBPβ staining of three independent experiments for each cell type. (b) ATF4 shows specific nuclear location in non-tumorogenic cells. Representative images, of three independent experiments, of ATF4 immunostaining. (c) ChoKIs induce PDI accumulation in tumor cells. The figure shows representative images of three independent experiments for each cell type, acquired with confocal microscopy for PDI immunostaining (green), after 18 and 24 h of ChoKIs. Nuclei were stained with DAPI in all cases.
CHOP mediates the cytotoxic effect of ChoKIs in breast cancer cells. As treatment with ChoKIs induces high levels of CHOP, we next assessed whether CHOP has a role in the cytotoxic effect of these inhibitors. CHOP siRNA was used to efficiently silence the protein in both MDA-MB231 and T47D cells (Figure 5a and Figure 5b, respectively). Flow cytometry analysis showed that ChoKIs did not promote cell death in CHOP silenced cells compared with controls (Figure 5c). Therefore, the induction of CHOP by ChoKIs has a main role in the cytotoxic effect on tumor cells.

TRB3 has been described as a target gene of CHOP and as mediator of ER stress-induced apoptosis in several cell types.\(^43\) We next asked whether CHOP regulated the overproduction of TRB3 induced by ChoKIs. TRB3 levels were found unaltered independently of the CHOP levels (Figure 5d), suggesting that TRB3 overproduction is not regulated by CHOP after ChoKIs treatment. The contribution of TRB3 to the cytotoxic effect caused by ChoKIs was also evaluated. TRB3 interference did not significantly modify the induction of cell death by either RSM932A or MN58b (Figure 5e), and it is not directly involved in cell death induced by ChoKIs.

ATF4 has a survival role in non-tumorigenic cells. Although ChoKIs induced ATF4 mRNA overexpression in tumor and non-tumorigenic cells, its protein level and nuclear location were only increased in the non-tumorigenic cells. We assessed whether this protein could participate in the cytostatic effect of ChoKIs in MCF10A. To that end, cells were transiently transfected with ATF4 siRNA and 24 h later treated with ChoKIs for 24 h (Figure 6a). Treatment with any of the ChoKIs increased cell death in ATF4 silenced cells (Figures 6b and c). These results demonstrate a crucial role of ATF4 in survival and protection of non-tumorigenic cells treated with RSM932A or MN58b.

Figure 4  (a) ChoK\(_x\) siRNA does not increase CHOP in T47D and MDA-MB231 cells. Cells were transiently transfected with ChoK\(_x\) siRNA or a non-targeted siRNA for 24 h. Afterwards, non-targeted siRNA-transfected cells were treated with ChoKIs for 24 h. Left panel shows protein levels of ChoK in T47D (a) and MDA-MB231 (b). Right panel shows CHOP protein levels after ChoK\(_x\) siRNA transfection. Data are shown as mean ± S.E.M. of four independent experiments. *P < 0.05 versus non-targeted siRNA-transfected control cells. (c) ChoK\(_x\) silencing diminishes CHOP overproduction caused by RSM932A. Cells were transiently transfected with 30 nM of ChoK\(_x\) siRNA or a non-targeted siRNA for 48 h, and then treated with RSM932A. Figure shows data as mean ± S.E.M. of three independent experiments and a representative gel showing the levels of ChoK\(_x\) or CHOP. GAPDH is shown as loading control. #P < 0.05 versus non-targeted siRNA + RSM932A-treated cells.
Figure 5  CHOP silenced tumor cells failed in the cytotoxic effect induced by ChoKIs. Cells were transiently transfected with 30 nM of CHOP SMARTPOOL siRNA or a non-targeted siRNA for 24 h. After that, cells were treated with ChoKIs for 24 h. Panels (a and b) show CHOP protein levels in MDA-MB231 and T47D, respectively. The graph bars show data as mean ± S.E.M. of three independent experiments and representative immunoblots. #P < 0.05 versus non-targeted siRNA + RSM-932 A-treated cells. $P < 0.05 versus non-targeted siRNA + MN58b-treated cells. (c) CHOP silenced tumor cells failed in apoptosis induced by ChoKIs. The graph bars show cell death as mean ± S.E.M. of three independent experiments. *P < 0.05 versus non-targeted siRNA-transfected control cells. (d) TRB3 overexpression caused by ChoKIs is not regulated by CHOP. Figure shows TRB3 protein levels in T47D transiently transfected with CHOP SMARTPOOL siRNA or a non-targeted siRNA for 24 h and then treated with ChoKIs for 24 h. Graph bars show data as mean ± S.E.M. of three independent experiments and a representative gel. (e) TRB3 interference did not affect the cell death induction by ChoKIs in T47D tumor cells. TRB3 was downregulated by shRNA pLKO.1 lentiviral system. Figure shows on the left panel TRB3 gene expression as mean ± S.E.M. of three independent experiments and on the right panel cellular viability percentage shown as mean ± S.E.M. of three experiments each one performed by quadruplicated.

Discussion

The relevance of ChoKα in tumor growth has been reported in some types of cancer where this enzyme was found to be overexpressed with high incidence. ChoKα-specific inhibitors have been generated with proven antiproliferative activity in vitro and in vivo. The antitumor action of ChoKIs relies on the ability of these compounds to directly affect cellular viability through induction of apoptosis. In contrast, in non-tumorigenic cells the blockage of de novo PCho synthesis by MN58b results in a reversible cell cycle arrest at G0/G1 phase. Here, we further confirm that MN58b and RSM932A exert cytotoxic effect in a variety of tumor cells and a cytostatic effect inducing G1 cell cycle arrest in non-tumorigenic mammary cells and normal derived colon mucosa primary cells. ChoKIs induced a marked deficiency in G1 to S phases.
versus non-targeted siRNA-transfected cells have been correlated with pathological states and particularly stress in carcinogenesis, and alterations in ER homeostasis functions. Recent studies suggest an important role of ER cellular processes required for cell survival and normal cellular investigated. stress apoptosis mediated by necrosis should be further ER stress activation of ChoKIs. Whether ChoKIs induce ER that these proteins are being ripped off as a consequence of showed pro-caspase 3 and PARP degradation, suggesting that these proteins are being ripped off as a consequence of, arguing that it contributes to survival or growth of some cancer cells. The UPR is primarily an adaptive response to support cell survival by a selective induction of transcription, but if homeostasis cannot be re-established, the UPR triggers cell death. According to this, some antitumor agents, including vorinostat, sorafenib, tocotrienol, delta(9)-tetrahydrocannabinol, fenretinide and bortezomib lead to apoptosis induced by ER stress. The ability of the cells to handle this stress, and transcriptional and translational regulation of ER stress mediators, may therefore condition their intrinsic capacity to adapt for cell survival or, alternatively, to start the apoptotic program through ER-associated machineries. In this sense, ChoKIs activate ER stress maintained response in tumor cells, but a slight ER proteins production was observed in non-tumor cells, indicating a different ability in both tumor and non-tumor cells to re-establish ER homeostasis disturbed by ChoKIs.

The results obtained after ChoKIs treatment of non-tumorigenic cells indicate that these cells are prone to ER stress adaptation through ATF4 overproduction and moderated increase of CHOP and C/EBPβ. In fact, ATF4 nuclear location was only observed in non-tumorigenic cells, suggesting that only in these cells it is activating its target genes. In keeping with this, inhibition of ATF4 expression blocked
proliferation and survival of non-tumorigenic cells. Moreover, HEK293 cells overexpressing ATF4 showed retarded growth in complete medium.50 We have observed increased cell death in non-tumorigenic cells silenced for ATF4 after treatment with ChoKIs, supporting its protective and pro-survival role.

Recent studies suggest that when a stress transcends from acute to persistent, the regulatory mechanisms based on UPR would promote sustained elevated levels of CHOP protein (despite of its short half-life), and the products of its target genes, triggering apoptotic pathways.32 CHOP is ubiquitously expressed at very low levels, and markedly expressed by perturbations that induce stress in a wide variety of cells.41,55 Upon non-stressed conditions, CHOP remains in the cytosol, and stress leads to its accumulation in the nucleus.41 The overinduction of ER stress response observed after ChoKIs treatment is not cell type specific as ChoKIs trigger this process in several types of breast, colon and lung cancer cells. CHOP protein levels were significantly increased at all times studied and accompanied by a significant increase of C/EBPα, GRP78, IRE1α, TRB3 and PDI, well-known markers for ER stress. In addition, ChoKIs also recruit CHOP and C/EBPα in the nuclei at the same time, promoting its dependent transcriptional program in tumor cells. We show a sustained significant increase of CHOP, mainly located in the nuclei. In addition, CHOP blockage using specific siRNA, abolished apoptosis induced by ChoKIs in tumor cells. CHOP silencing also induced a reduction on C/EBPα, ATF3 and GRP78 protein levels (data not shown), which in addition are also direct inducers of CHOP. Cells lacking C/EBPα (major partner of CHOP) are also resistant to ER stress-induced apoptosis.42 Thus, a specific, potent, overstated and persistent induction of ER proteins by ChoKIs drives the tumor cells to apoptosis mediated by elevated levels of CHOP.

TRB3 has been described as a novel ER stress-inducible gene that is a direct transcriptional target of ATF4 and CHOP and may have an important role in ATF4/CHOP-mediated apoptosis.43 Knockdown of ATF4 or CHOP significantly suppressed the induction of TRB3 in HEK293.50 Conversely, no change was observed in TRB3 levels in CHOP silenced cells treated with RSM932A or MN58b, indicating that in this case TRB3 is not directly involved in the cytotoxic effect of high levels of CHOP. The implication of TRB3 in the mechanism of action of ChoKIs has to be more deeply investigated.

PC depletion can disturb the ER function and result in ER stress.32,53 Inhibition of PC synthesis through mutated CTP:phosphocholine cytidylyltransferase enzyme leads specifically to induction of the ER stress-related protein CHOP and apoptosis.50 Moreover, the C/EBPα-ATF composite sites are required for the increased expression of CHOP during PC depletion and might be mediated by binding of ATF2 to this element.53 The specific effect of ChoKIs is not observed after siRNA ChoKα inhibition. Specific pharmacological inhibition and ChoKα siRNA efficiently reduced ChoKα activity and PCho levels,15-18 but ChoKα siRNA failed in the activation of ER stress response. The different strategies to block ChoKα by siRNA or specific pharmacological inhibition might explain this differential effect. Our results indicate that ER stress response triggered by ChoKIs depends on the presence of ChoKα, as the higher the silencing of ChoKα protein, the lesser the induction of CHOP was observed after ChoKIs treatment. In vitro binding experiments suggest that ChoKα conformation might be modified after ChoKIs interaction, increasing the potential of apoptosis induction of this family of inhibitors by combining a metabolic effect by depleting PC synthesis, and a UPR response. These results are consistent with a dual effect of ChoKIs and are in keeping with recent reports demonstrating a complex interaction of ChoKα with its inhibitors.54,55

Accumulation of proteins with wrong conformations or unfolded proteins in the ER results in the induction of several proteins, including the ER chaperones GRP78 and GRP94, which facilitates proper protein folding by interacting with exposed hydrophobic patches on protein-folding intermediates and is thought to prevent their aggregation while maintaining the protein in a folding-competent state.32,56 Furthermore, ER stress can activate transmembrane proteins located in the ER membrane like IRE1α and PERK, which inhibit general protein synthesis and activate the non-canonical apoptosis response. ChoKIs dramatically increase GRP78, IRE1α and CHOP, which suggests that the exacerbated induction of ER stress response depends on ChoKα protein presence in the cells, and the levels of ChoKα are crucial for the ER stress-mediated apoptosis by these inhibitors. Furthermore, non-cytotoxic doses of both MN58b and RSM932A showed a significant increase of CHOP in a dose-dependent manner (data not shown), which dismisses that it is an off-target effect and support that ER stress induction caused by ChoKIs may be directly related to the inhibition of ChoKα activity.

In summary, ChoKα inhibition leads to exacerbated ER stress response and CHOP overproduction that has a crucial role in the cytotoxic effect of ChoKIs in tumor cells. In addition, ATF4 might represent a pathway for cell survival in non-tumorigenic cells. Our data provide relevant evidence for a rational understanding of the selective and specific apoptotic effect on tumor cells, and support further preclinical and clinical studies with this novel family of anticancer drugs.

Materials and Methods
Cell culture and reagents. Cell lines were grown under standard conditions: T47D (epithelial mammary cells from ductal carcinoma), MCF7 (epithelial mammary gland cells from adenocarcinoma), H460 (epithelial large cell non-small-cell lung cancer); were cultured in RPMI (Invitrogen, Casbard, CA, USA); MDAMB231 (epithelial mammary gland cells from adenocarcinoma) and SW620 (colorectal adenocarcinoma), cultured in DMEM (Invitrogen), supplemented all of them with 10% fetal bovine serum (Invitrogen), MCF10A (non-tumorigenic epithelial mammary gland mammary cell line) were grown in DMEM-F12 (Invitrogen) supplemented with 10% newborn calf serum (Biochrom, Berlin, Germany), 10 µg/ml Epidermal growth factor (Calbiochem, Merck, Darmstadt, Germany), 50 µg/ml hydrocortisone, 2.5 mg/ml insulin and 100 ng/ml choloro toxin (from Sigma-Aldrich, St. Louis, MO, USA). NCM460 cells (normal derived colon mucosa cells) were received by a licensing agreement with INCELL Corporation (San Antonio, TX, USA). They were routinely propagated on M3BASE medium plus supplements and 10% FBS. All experiments were performed at 50% of confluence.

First- and second-generation inhibitors of ChoKα were used. MN58b was dissolved in water at 5 mmol, and RSM932A was dissolved in DMSO: Water (2:1) at 5 mmol. The vehicle DMSO: Water (2:1) were used as Control. Both drugs are produced at Prof. Lacal’s laboratory and are not available as commercial compounds.

Flow cytometry analysis. Cells were seeded at a density of 2 × 10^4 cells/well on six-well plates and treated 24 h later with MN58b and RSM932A.
Supernatants and cultured cells were collected and centrifuged at 1800 r.p.m. for 15 min. Then cells were diluted in 1 x PBS with PI (50 µg/ml), RNase A (20 µg/ml) and TX-100 (50 µl). The DNA content of stained cells was analyzed using a FACScan flow cytometer (Becton Dickinson and Company, Franklin Lanes, NJ, USA) and Cell Quest Pro software (Becton Dickinson and Company). The fraction of cells in apoptosis (sub-G1), G0-G1, S and G2-M phases were quantified by MODFIT LT 3.0 software (Verity Software House, Topsham, ME, USA).

Cell viability assays (MTT). Cells were seeded at a density of 5 x 10^3 cells/well on 96-well plates. Then, cells were treated with MN58b or RSM932A and incubated with pro-caspase 3; cycin D1, phospho-RB, RB (Santa Cruz Biotechnology, Heidelberg, Germany), CHOP, GRP78 and IRE1α (Cell Signaling, Danvers, MA, USA), C/EBPβ, TRB3, ATF4, ATF3 (AbCam, Cambridge, UK) and Chokx antibody. α-Actin (Sigma-Aldrich) and GAPDH (Millipore, Billerica, MA, USA) were used as loading controls. Membranes were incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed using an ECL chemiluminescence kit (Amer sham Pharmacia Biotech, Piscataway, NJ, USA). Autoradiographs were scanned and Quantity One software was used for densitometric analyses (Bio-Rad, Madrid, Spain).

Gene expression studies. Total mRNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using high Capacity cDNA Archive Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using 1 µg of total RNA primed with random hexamer primers following the manufacturer’s instructions. Real-time RT-PCR was performed using fluorogenic TaqMan gene expression assays (Applied Biosystems): CHOP (HS_01090850_m1), C/EBPβ (HS_00270923_s1), ATF4 (HS_0090569_g1) and TRB3 (HS_00221754_m1). Data were normalized with 18S eukaryotic ribosomal RNA expression (HS9999990_1). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value (’arithmetic fit point analysis for the lightcycler’). Results are expressed as the quantity of the target gene after treatment relative to unstimulated cells, after normalization against 18S.

siRNA and shRNA transfection studies. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) and 30 nM siRNA for ATF4 (Eurogentec S.A., Liege, Belgium), CHOP on-target plus SMARTpool CHOP (Dharmacon Inc., Chicago, IL, USA), Chokx (Qiagen) and non-targeted siRNA as control (Ambion, Life Technologies, Carlsbad, CA, USA) as described by the manufacturer’s instructions. Lentiviruses were generated by co-transfecting Hek293T cells with Lentivirus Production Kit (Dharmacon Inc.) and selected in 1 µg/ml of TRB3 shRNA-encoding plasmid (Dharmacon Inc.) and non-targeted siRNA as control (Ambion, Life Technologies, Carlsbad, CA, USA) as described by the manufacturer’s instructions. Lentiviruses were generated by co-transfecting HEK293T cells with 10 µg of TRB3 shRNA-encoding plasmid (Dharmacon Inc.) and 8.75 µg of pDelta 8.9 (psPAX2 and VSV-G (pMD.G2) plasmids using Lipofectamine 2000 (Invitrogen). Growth media were exchanged the following day and lentivirus-containing supernatant was harvested 48h later. Target cell lines were transduced with lentiviral RNAi at MOI = 1 and selected in 1 µg/ml puromycin for 1 week.

Native gel analysis. Ten micromolar samples of Plasmidium falciparum and human choline kinase were incubated with DMSO or 100 µM of RSM932A in Kinase Buffer (100 µM Tris, pH 8, 10 mM MgCl2) for 1 ½h on ice and after the addition of an appropriate loading buffer were loaded onto a 4–20% Mini-Protean TGX native gel (Bio-Rad).

Trypsin degradation. Ten micromolar samples of P. falciparum and human choline kinase were incubated with either DMSO or 100 µM of RSM932A in Kinase Buffer in the presence of 3 µg/ml of trypsin at 37 degrees for 1 h. Samples aliquots were removed at 5, 10, 20, 30 and 60 min, and placed on ice. Sample buffer was immediately added and trypsin degradation reaction was stopped by incubating for 5 min at 95 degrees. Samples were then loaded onto a 12% SDS-PAGE gel. Coomassie-stained bands representing undegraded choline kinase were quantified using the Quantity One software and normalized against a band representing a sample lacking trypsin.

Immunofluorescence staining. To assess CHOP, C/EBPβ, ATF4 and PDI staining, cells were fixed with 4% PFA. After permeabilizing with 0.3% TX-100, samples were blocked with 4% BSA and incubated with primary antibodies: PDI, CHOP (Cell Signaling), C/EBPβ and ATF4 (Santa Cruz Biotechnology). After washing, cells were incubated with Alexa secondary antibodies (Invitrogen), and nuclei were stained with DAPI (Invitrogen). Negative control was performed in the absence of primary antibody or using non-related secondary antibodies. Samples were mounted with ProLong gold (Invitrogen) and examined using TSC SP2 confocal microscope (Leica, Barcelona, Spain).

Statistical analysis. One-way ANOVA was used to compare gene and/or protein expression levels between groups. When statistical significance was found, the Bonferroni post hoc comparison test was used to identify differences between groups. Differences were considered significant at P < 0.05. Statistical analyses were performed using the SPSS statistical software, version 11.0.

Conflict of Interest

The authors declare no conflict of interest.

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