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► To cite this version:

Charlotte Dubois, Artem Kondratskyi, Gabriel Bidaux, Lucile Noyer, Eric Vancauwenberghe, et al.. Co-targeting Mitochondrial Ca²⁺ Homeostasis and Autophagy Enhances Cancer Cells' Chemosensitivity. *iScience*, 2020, 23 (7), p.101263. 10.1016/j.isci.2020.101263 . hal-03060381

HAL Id: hal-03060381

<https://hal.science/hal-03060381>

Submitted on 13 Dec 2020

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CO-TARGETING MITOCHONDRIAL Ca^{2+} HOMEOSTASIS AND AUTOPHAGY ENHANCES CANCER CELLS CHEMOSENSITIVITY

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PII: S2589-0042(20)30449-1

DOI: <https://doi.org/10.1016/j.isci.2020.101263>

Reference: ISCI 101263

To appear in: *ISCIENCE*

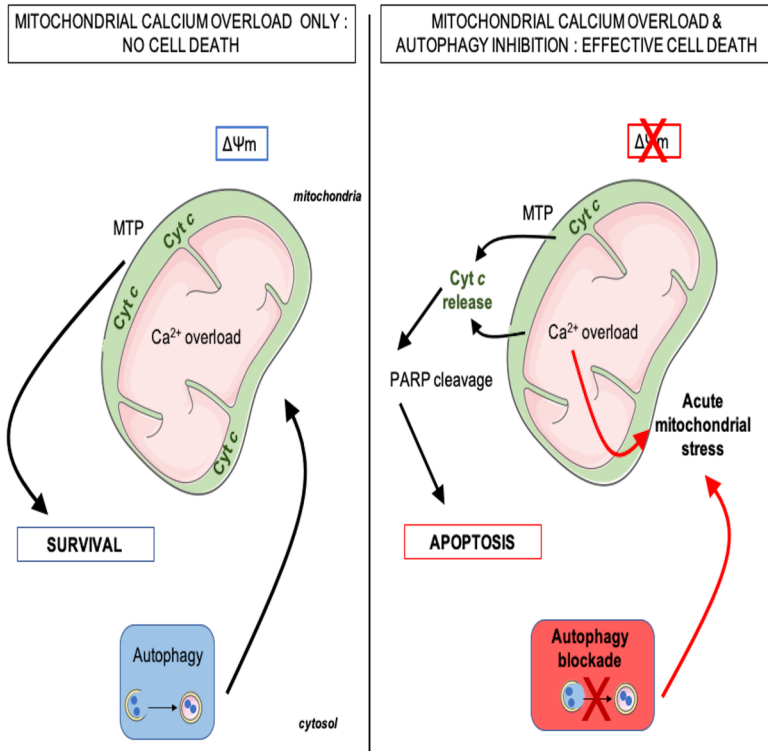
Received Date: 16 March 2020

Revised Date: 17 April 2020

Accepted Date: 8 June 2020

Please cite this article as: Dubois, C., Kondratskyi, A., Bidaux, G., Noyer, L., Vancauwenberghe, E., Farfariello, V., Toillon, R.-A., Roudbaraki, M., Tierny, D., Bonnal, J.-L., Prevarskaya, N., Abeele, F.V., CO-TARGETING MITOCHONDRIAL Ca^{2+} HOMEOSTASIS AND AUTOPHAGY ENHANCES CANCER CELLS CHEMOSENSITIVITY, *ISCIENCE* (2020), doi: <https://doi.org/10.1016/j.isci.2020.101263>.

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**CO-TARGETING MITOCHONDRIAL CA²⁺ HOMEOSTASIS AND AUTOPHAGY
ENHANCES CANCER CELLS CHEMOSENSITIVITY**

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Subject areas: Calcium, apoptosis, mitochondria, autophagy, chemoresistance, *ex-vivo* tumor models, xenograft mouse models, combined therapy.

Journal Pre-proof

Summary

Mitochondria are important cell-death checkpoints and mitochondrial Ca^{2+} overload is considered as a potent apoptotic intrinsic pathway inducer. Here, we report that this Ca^{2+} apoptosis link is largely ineffective in inducing cell-death just by itself and required a concomitant inhibition of autophagy to counteract its pro-survival action. In such condition, an acute mitochondrial stress revealed by a DRP1 mediated mitochondrial dynamic remodeling is observed concomitantly with mitochondrial depolarization, release of cytochrome *c* and efficient apoptosis induction. We also uncover that mitochondrial Ca^{2+} status modulates the function of autophagy as a sensitizer for chemotherapies. This priming mediated by mitochondrial Ca^{2+} overload and inhibition of autophagy sensitizes many cancer cells types to different chemotherapies with independent mechanisms of action. Collectively, our results redefine an important cell signaling pathway, uncovering new combined therapies for the treatment of diseases associated with mitochondrial Ca^{2+} homeostasis disorders such as cancer.

Introduction

Calcium (Ca^{2+}) is a critical intracellular second messenger, which controls key cell fate decisions, such as metabolism, proliferation or apoptosis (Rossi et al., 2019). Specific Ca^{2+} signaling pathways have long been known to play an important role in apoptosis induction and/or regulation, which occurs through endoplasmic reticulum (ER) Ca^{2+} stress, cytosolic and mitochondrial Ca^{2+} overload (Berridge et al., 2000; Orrenius et al., 2003; Pinton et al., 2008). Each of these pathways has been associated alone or in combination with apoptosis induction or resistance, both in physiological and physiopathological conditions. Indeed, the ER lumen is the major storage of intracellular Ca^{2+} , allowing the proper folding of proteins by Ca^{2+} -binding chaperones (Wang and Kaufman, 2014). Depletion of ER Ca^{2+} stores is considered to be a robust stress condition that triggers cell death (Giorgi et al., 2010; Prevarskaya et al., 2004). Other studies have also demonstrated that a large and sustained elevation of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) is another key process for triggering apoptosis, mainly induced by store-operated Ca^{2+} entry (SOCE) (Vanden Abeele et al., 2002; Prevarskaya et al., 2010). Finally, mitochondrial Ca^{2+} overload resulting from sustained elevation of $[\text{Ca}^{2+}]_c$ is considered to be one of the ways to induce mitochondrial-mediated intrinsic apoptotic pathway (Berridge et al., 2000; Orrenius et al., 2003; Pinton et al., 2008; Zhivotovsky and Orrenius, 2011). The crucial factor is the magnitude of the Ca^{2+} signal received by the mitochondria. A high Ca^{2+} load will lead to the mitochondrial permeability transition pore (MPTP) (Lemasters et al., 2009) and eventually to the permeabilization of the outer mitochondrial membrane (OMM). As a consequence, targeting mitochondria and in particular mitochondrial Ca^{2+} homeostasis to sensitize cancer cells to apoptosis and to overcome drug resistance, represents a major aim in anti-cancer therapy (Wen et al., 2013; Zhang et al., 2016).

However, the expected role of Ca^{2+} could be less clear than it has first been proposed. Indeed, we have identified in a previous study, that a potent Ca^{2+} homeostasis disruption

characterized by a sustained increase of cytosolic Ca^{2+} and endoplasmic-reticulum (ER) Ca^{2+} stress is not sufficient enough to induce apoptosis (Dubois et al., 2013). However, the role of mitochondria was not investigated and more importantly the molecular mechanisms underlying this atypical mode to escape from cell-death were not identified. Moreover, an increasing number of studies have demonstrated that Ca^{2+} also plays an important role in the regulation of more recent hallmark of cancer, such as autophagy involved in tumor development and cancer therapy (Degenhardt et al., 2006; Hanahan and Weinberg, 2011; Yang et al., 2011). Autophagy is a catabolic degradation process in which cellular proteins and organelles are enveloped by double-membrane autophagosomes and degraded in lysosomes (Chen and Klionsky, 2011; Ravikumar et al., 2010). The autophagic process attempts to restore metabolic homeostasis and the current consensus is that autophagy has a dual role in cancer (Jiang et al., 2015; Kroemer, 2015; White, 2015). During the early steps of cancer development, autophagy functions as a tumor suppressor mechanism by preventing the accumulation of damaged organelles and aggregated proteins. In contrast, autophagy is considered as a pro-survival mechanism for established tumors in response to metabolic stresses such as nutrient deprivation, hypoxia, and absence of growth factors. Autophagy has also emerged as a crucial player in drug resistance in response to chemotherapies (Kroemer, 2015). Importantly, autophagy is shown to precede or to act in parallel with apoptosis process but the precise molecular mechanisms involved are still not fully understood (Sui et al., 2013) and in particular the role of autophagy in cell-death mediated by Ca^{2+} homeostasis disruption is not fully understood. Finally, it has been also reported that some Ca^{2+} signaling pathways and Ca^{2+} modulators used to depict apoptotic pathways have also significant implications in other signaling pathways such as autophagy, highlighting the need of better tools (Bootman et al., 2018; Dubois et al., 2016).

Here, we dissect the interplay between survival pathways such as autophagy and mitochondrial dynamics to establish their precise role(s) in cell death mediated by Ca^{2+} homeostasis disruption. Collectively, our results challenge a crucial paradigm in cell death and provide new concepts for more rational approaches in cancer treatments. Indeed, we propose an effective combined strategy based on concomitant mitochondrial Ca^{2+} overload and autophagy inhibition with drugs in clinical evaluation to increase chemotherapies sensitivity in most common cancer types.

Journal Pre-proof

Results

Ca²⁺ homeostasis perturbations *per se* are not sufficient to induce apoptotic cell death

To investigate the role of Ca²⁺ homeostasis perturbations in apoptosis induction, we performed an in-depth study by using several sarcoplasmic endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitors (Thapsigargin (TG), TG analogues and cyclopiazonic acid (CPA)). SERCA resides in the endoplasmic reticulum (ER) and plays a crucial role in maintaining cellular Ca²⁺ homeostasis (Berridge et al., 2000; Orrenius et al., 2003; Pinton et al., 2008). We used concentrations of SERCA inhibitors known to induce a robust disruption of Ca²⁺ homeostasis (Quynh Doan and Christensen, 2015). The TG analogues tested were ASP-8ADT (also known as 12-ADT-ASP), EPO-8ADT and LEU-8ADT, and all of them have been characterized in cell free system for their specific activity on SERCA pumps and functionally on several cell types (Jakobsen et al., 2001). As we have already demonstrated (Dubois et al., 2013), all analogues were able to induce an influx of Ca²⁺ across the plasma membrane due to the activation of SOC (Store Operated Channel) in response to ER Ca²⁺ store depletion triggered by SERCA inhibition (Figure 1A). Here, we therefore evaluated their ability to maintain high cytosolic Ca²⁺ concentration ([Ca²⁺]_c) over a longer period of time which has been also associated with apoptosis induction (Figure 1B) (Berridge et al., 2003; Clapham, 2007). We observed that only TG, ASP-8ADT and LEU-8ADT (1 μM/ 18 hr) were able to induce a sustained [Ca²⁺]_c increase compared to EPO-8ADT or CPA (1 μM/ 18 hr) (Figure 1B). In accordance, these discrepancies may have significant consequences on mitochondria Ca²⁺ contents, as it is largely accepted that sustained Ca²⁺ entry is also a major factor in the process of mitochondrial Ca²⁺ overload (Berridge et al., 2000; Orrenius et al., 2003; Pinton et al., 2008; Zhivotovsky and Orrenius, 2011). Thus, we evaluated the extent of mitochondrial Ca²⁺ overload following the same setting of treatment. We observed that, only

TG, ASP-8ADT and LEU-8ADT induced mitochondrial Ca^{2+} overload in prostate cancer cell lines (Figures 1C, D) and 5 additional cancer cell lines (Supplementary Figure 1). In parallel, we correlated the ability of these compounds to induce Ca^{2+} mediated apoptosis in prostate (Figures 1E, F, G, H), breast (Figure 1I) and pancreatic cancer cells (Figure 1J) (Supplementary Figure 1A-O). Surprisingly, only TG and LEU-8ADT were potent apoptosis inducers in all cell lines. Both compounds have a similar ability to induce mitochondrial Ca^{2+} overload and to maintain high $[\text{Ca}^{2+}]_c$ over a longer period (Figure 1B). However, LEU-8ADT induced a slower initial Ca^{2+} influx rate compared to TG (Figure 1A). Conversely, the initial Ca^{2+} influx rate was higher for ASP-8ADT compared to LEU-8ADT despite its low efficiency in inducing cell death. This implies that the initial Ca^{2+} influx was not correlated with the induction of apoptosis. Concerning TG and ASP-8ADT, while the remodeling on Ca^{2+} homeostasis was quite similar, compared to TG, apoptosis induced by ASP-8ADT was very modest and particularly in the LNCaP or PC-3 prostate cancer cell lines. These results were unexpected as they are not in line with the commonly established key role of mitochondrial Ca^{2+} overload in the process of apoptosis induction. Thus, we decided to confirm that ASP-8ADT was able to induce mitochondrial Ca^{2+} overload by using two other independent approaches that allow a direct assessment of Ca^{2+} handling by mitochondria. By measuring steady-state Ca^{2+} concentration in mitochondria with the 4mtD3cpv biosensor or with the ratiometric genetically encoded Ca^{2+} probe mito-GEM-GECO1, we confirmed that TG and ASP-8ADT induced similar mitochondrial Ca^{2+} overload (Figure 1K) (Supplementary Figures 1P-R and methods section).

Importantly, at the micro-molar concentration (1 μM) used in the present study, both TG and ASP-8ADT totally inhibit SERCA activity (Winther et al., 2010). Thus, differences observed in apoptosis induction were not correlated to differential action or affinity on SERCA activity. Moreover, in a previous study (Dubois et al., 2013), we already observed that ASP-8ADT did

not cause as much cell death during chronic exposure and we attributed this effect to a weaker ability of the ASP-8ADT compound to keep the ER stores depleted. However, this conclusion is ruled out in the present study, as we were unable to detect differences in the hallmarks of Ca^{2+} changes induced by TG or ASP-8ADT over a longer period covering the whole duration of apoptosis induction phase. We also checked that the uncoupling between Ca^{2+} homeostasis disruption and apoptosis induction did not result from impairments in ER stress response (Supplementary Figures 1S-Y) or Reactive Oxygen Species (ROS) accumulation in response to mitochondrial Ca^{2+} overload mediated by ASP-8ADT (Figure 1L). This in-depth study suggests the involvement of an undefined step/factor needed for an efficient cell death induction in response to Ca^{2+} homeostasis disruption and following mitochondrial Ca^{2+} overload.

Mitochondrial Ca^{2+} overload induces a partial MPTP opening, ineffective to induce mitochondrial membrane depolarization and cyt *c* release

It is generally considered that mitochondrial Ca^{2+} overload induces continuous MPTP openings during cell death leading to loss of both Ca^{2+} and proton gradients across the inner mitochondrial membrane (Baumgartner et al., 2009; Giorgi et al., 2012). We used the gold standard calcein/ Co^{2+} -quenching technique, which allows a quantification of MPTP opening (Bonora et al., 2016). We showed that a 18 hr pretreatment with either TG (known to promote MPTP opening (Korge and Weiss, 1999)) or ASP-8ADT, were associated with a similar cobalt-mediated quenching of calcein fluorescence (Figures 2A, B) suggesting that MPTP was opened to an equivalent extent. This MPTP opening was abolished by a 1hr-pretreatment with cyclosporine A (CsA; 0,5 μM), an inhibitor of MPTP opening which binds cyclophilin D. We also tested ionomycin (IM; 1 μM), a Ca^{2+} ionophore known to trigger mitochondrial Ca^{2+} overload and activation of MPTP in different cell models (Chaudhuri et al., 2016; Ying et al.,

2018). We observed with IM a potent activation of MPTP compared to TG and ASP-8ADT (Figure 2C). In accordance, CsA pre-treatment (1 hr before TG or ASP-8ADT treatments; 0,5 μ M) induced an increase of mitochondrial Ca^{2+} content in response to TG or ASP-8ADT (Figure 2D). We concluded from these experiments that both ASP-8ADT and TG were able to induce similar but not maximal MPTP opening (as observed with IM). These experiments challenge the role of MPTP openings and mitochondrial Ca^{2+} overload implying that another process impaired cell death induction with ASP-8ADT. As a readout of cell death and MPTP openings, we evaluated the release of *cyt c* (Figures 2E, F) (Figures S2A, B) and the loss of mitochondrial membrane potential (Figures 2G, H). Indeed, *cyt c* release from mitochondria and loss of mitochondrial membrane potential ($\Delta\Psi_m$) are considered to occur subsequently to continuous pore activation. Our results clearly showed that ASP-8ADT failed to induce these features compared to TG and this, despite its ability to induce both similar mitochondrial Ca^{2+} overload and MPTP opening. We also clearly demonstrated that TG induced only a partial mitochondrial depolarization in comparison to the mitochondria uncoupler FCCP (1 μ M) (Figures S2C, D). Our results also demonstrated that IM is a more potent inducer of MPTP opening compared to either ASP-8ADT or TG (Figure 2C). Thus, this partial MPTP opening induced by ASP-8ADT may explain that mitochondrial membrane potential is preserved with ASP-8ADT. Indeed, MPTP opening is intrinsically depolarizing, but we clearly showed that only partial MPTP opening occurs. Moreover, a situation with no mitochondrial Ca^{2+} overload occurred only when mitochondrial potential is totally collapsed by using FCCP as pretreatment (Figure 2D). Taken together, these results suggest that partial MPTP opening resulting from mitochondrial Ca^{2+} overload and ROS accumulation is not enough to induce loss of mitochondrial membrane potential and release of *cyt c*.

Autophagy inhibition is a pre-requisite for mitochondrial depolarization leading to

apoptosis mediated by Ca²⁺ homeostasis disruption

TG-treated cells exhibited mitochondrial membrane depolarization and efficient apoptosis induction, suggesting a direct link with its apoptotic potency. Interestingly, it has been reported that TG has significant implications in other Ca²⁺-mediated and Ca²⁺-independent signaling pathways such as autophagy (Bootman et al., 2018; Decuyper et al., 2011). We hypothesized that apoptosis mediated by TG could be related to the inhibition of survival pathways such as autophagy. We used, an established strategy, a tandem-tagged GFP-mCherry-LC3 probe that can determine whether an autophagosome has fused with a lysosome, based on the distinct chemical properties of GFP and mCherry fluorophores (Kimura et al., 2007). Under non-lysosomal and near-neutral pH conditions, both GFP and mCherry fluoresce. However, the low pH in the lumen of the lysosomes quenches the GFP signal, but not the mCherry. Using this approach, we found that upon TG treatment (2 hr, 1 μ M) in LNCaP cells, multiple autophagosomes are visible without GFP quenching, thereby suggesting that fusion with lysosomes did not occur (Figures 3A, B). In contrast, upon ASP-8ADT treatment (2 hr, 1 μ M), only mCherry-positive structures have accumulated. The addition of chloroquine (CQ) (prevents endosomal acidification leading to an inhibition of lysosome-endosome fusion) in association with ASP-8ADT mimics the autophagic signature observed with TG. Indeed, by quantifying the size of acidic vesicles formed after treatment with TG, ASP-8ADT alone or in combination with CQ, we observed an increase in size upon ASP-8ADT when combined with CQ to the same extent as TG (Figure 3C). In a second attempt, we evaluated the action of ASP-8ADT on the autophagic flux in LNCaP cells by using Western Blot analysis of LC3 I and II (formation of autophagosome) and P62 (autophagic flux) (Figures 3D, E, F). These results confirmed previous studies that reported a block of autophagy by TG, as revealed by both accumulation of P62 and LC3 II. These effects were drastically reduced with ASP-8ADT, thus revealing its inability to interfere with the

autophagic process (Figures 3D, E, F) (see also Figure S3). We also confirmed the action of ASP-8ADT and TG on autophagic flux in PC-3 prostate cancer cells and we also evaluated their action on autophagic flux in conditions of autophagy induction induced by starvation (HBSS, 2 hr) (Figures S3A, B, C, D). These results confirmed that TG is able to block autophagy, as revealed by both accumulation of P62 and LC3 II. These effects were greatly reduced with the ASP-8ADT compound. We also confirmed by real-time PCR of P62 mRNA levels that P62 protein level in ASP-8ADT treated cells did not depend on variation of transcription (Figure S3D). We also checked the Beclin1 status (Figure S3E). We have further investigated the activity of TG on the autophagic process by using a more qualitative approach. By electron microscopy, we showed that only TG prevented the closure of the autophagosome and induced an accumulation of immature autophagosome that we never observed following ASP-8ADT treatment (Figure 3G).

We took advantage of the fact that ASP-8ADT did not exert an inhibitory effect on autophagy in LNCaP cells in order to study its implication on apoptosis induced by Ca^{2+} homeostasis disruption. We used a combined treatment with an ER Ca^{2+} stressor (TG or ASP-8ADT) and established autophagic inhibitors such as Chloroquine (CQ), Wortmanin (WT), Bafilomycin A1 (Baf A1) and a Rab7 inhibitory peptide. Hoechst staining experiments shown, using autophagy inhibitors, a 6-fold increase of ASP-8ADT potency to induce cell death (from 5% to 30%), without additional effect on TG potency after 48 hr of treatment (Figure 4A). By western blot we confirmed the cleavage of PARP (Figure S4F) as a marker of caspase-dependent apoptosis. We also performed immunostaining of cyt *c* on LNCaP cells treated with ASP-8ADT (1 μ M, 18 hr) alone or in combination with the autophagic inhibitor Rab7 (100 nM). Confocal images showed that ASP-8ADT induced the release of cyt *c* only upon autophagy inhibition (Figures S4A, B). We confirmed the requirement of autophagy inhibition with a genomic strategy by using siRNA against ATG5, which participates in the formation of au-

tophagosome (Ganley et al., 2011). Thereby, we showed that an early inhibition of the autophagic process by the down regulation of ATG5 leads to an increase in apoptosis induced by ASP-8ADT (Figure 4B). However, the percentage of apoptotic cells was not as high as with other inhibitors of autophagy (Figure 4A) or TG, because siRNA mediated knockdown of the autophagic regulatory gene ATG5 was only partial (Figure 4B, upper panel). By flow cytometry using the fluorescent mitochondrial membrane probe DiOC6(3), we demonstrated that Rab7 inhibitory peptide restored the ability of ASP-8ADT to induce mitochondrial membrane depolarization (Figure 4C). These results were confirmed using the JC-1 dye. We observed the progressive loss of red JC-1-aggregate fluorescence (polarized mitochondria) and a cytoplasmic diffusion of green monomer fluorescence (depolarized mitochondria) following exposure to ASP-8ADT in combination with Rab7 inhibitor (Figure 4D). We confirmed this specific point in prostate LNCaP, PC-3 and breast MCF-7 cancer cell lines (Figure 4D). Thus, these data clearly demonstrated that inhibition of autophagy is required for efficient mitochondrial membrane depolarization upon mitochondrial Ca^{2+} overload leading to the release of *cyt c* and apoptosis induction. Finally, we investigated the potential role of BAX and BCL-2 proteins, two crucial actors involved in the mitochondrial apoptotic pathway, by studying their expression and subcellular distribution at mitochondria in LNCaP cells. Using subcellular fractionation technique, we observed that BAX level was nearly the same at mitochondria following treatments with TG or ASP-8ADT alone or in combination with autophagy inhibition. In contrast, BCL-2 expression was higher in all the conditions compared to control (Figure 4E). These findings suggest that OMM permeabilization driven by BAX and BCL-2 interactions is not a critical event in apoptosis induced by TG or ASP-8ADT (in combination with autophagy inhibition). Thus, the weaker ability of ASP-8ADT to kill the cells could not be explained by the promotion of the BCL-2 survival pathway.

Concomitant inhibition of autophagy and mitochondrial Ca^{2+} overload is required for inducing an acute mitochondrial stress revealed by a DRP1 mediated mitochondrial dynamic remodeling

We further explored the consequences of mitochondrial Ca^{2+} overload and autophagy inhibition in the control of cell fate. It is well known that mitochondrial dynamics (fusion and fission) and autophagy, work together as a quality control mechanism in the life cycle of the mitochondrion (Twig et al., 2008). During the apoptotic process, mitochondrial networks are dramatically reorganized from long filamentous interconnected tubules into small punctate spheres. Whether remodeling of mitochondrial networks is necessary for apoptosis-associated *cyt c* release, or is merely an accompanying process, is still a subject of debate (Sheridan and Martin, 2010). 3D reconstruction from confocal imaging experiments clearly showed that the filamentous interconnected tubules of the mitochondrial networks were preserved with ASP-8ADT alone but disrupted when combined with autophagy inhibitor as observed with TG (Figures 4F, G). These results clearly showed that mitochondrial Ca^{2+} overload alone did not induce significant changes in mitochondrial dynamics suggesting that cell fate was not compromised when autophagy can play its pro-survival role. On the contrary, concomitant inhibition of autophagy forced the cell to use another pro-survival pathway mediated by mitochondrial dynamics. Indeed, recent studies have demonstrated that under sustained stress conditions, a DRP1-dependent mitochondrial fission is triggered and is involved in cellular survival at the early stage of injury (Ikeda et al., 2014; Zuo et al., 2014). In this context, we showed that siRNA-mediated knockdown of DRP1 restored the apoptotic potential of ASP-8ADT (Figure 4H) confirming the crucial role of mitochondrial dynamics. siDRP1 treatments were functionally validated by confocal microscopy using MitoTracker Red CMXRos staining (via the observation of the characteristic mitochondrial hyperfusion) and western blot (Figure S4C, D, E). These results showed that a mitochondrial dynamic remodeling mediated

by DRP1 is triggered and is involved in cell survival only when cells were exposed to concomitant Ca^{2+} homeostasis disruption and autophagy inhibition. However, this pro-survival mechanism is not sufficient to prevent apoptosis induction induced by TG or ASP-8ADT in combination with autophagic inhibitors. These results also pointed out that autophagy and fission/fusion mitochondrial dynamics are two processes that could be used by the cell to counteract the mitochondrial stress mediated by Ca^{2+} overload. These pro-survival pathways prevent mitochondrial collapse by inhibiting mitochondrial membrane depolarization. In fact, mitochondrial membrane depolarization seems to be the last and decisive event preceding apoptosis in response to mitochondrial Ca^{2+} overload. We confirmed this feature by using FCCP (0,5 μM , 48 hr) to induce mitochondrial membrane depolarization and indeed, FCCP restored the ability of ASP-8ADT to induce cell death, whereas used alone it failed to do this significantly (Figures 4I, J).

Dual targeting of Ca^{2+} homeostasis and autophagy improves chemotherapy efficiency

in vitro and in vivo

Targeting mitochondria or autophagy to sensitize cancer cells to apoptosis is a major aim in anti-cancer therapy (Jiang et al., 2015; Kroemer, 2015; Wen et al., 2013; White, 2015; Zhang et al., 2016) and the medical and scientific community has also come to accept that monotherapy is unlikely to be the answer to completely destroy cancer cells (Hanahan, 2014). Our results confirm that targeting mitochondrial Ca^{2+} homeostasis alone displays only poor efficiency. The molecular basis underlying this lack of efficiency is that apoptosis mediated by mitochondria Ca^{2+} overload requires inhibition of autophagy. This new interplay challenges a crucial paradigm in cell death and our results reveal novel combinatorial therapeutic strategies to improve clinical outcomes.

ASP-8ADT is the active compound of the Mipsagargin family, a promising new anticancer treatment approved by the Food and Drug Administration (FDA) and used in several clinical trials (NCT01056029 and NCT01734681)) (Denmeade and Isaacs, 2005; Mahalingam et al., 2016). Unfortunately, results are below expectations as published in a recent report (Mahalingam et al., 2016). Based on our fundamental findings we have evaluated using xenograft mouse models, cell line-based functional studies, and *ex-vivo* tumor models from clinical specimens, the relevance of our combined strategy to (1) increase the efficiency of ASP-8ADT *in vivo* (2) sensitize cancer cells to chemotherapies using a co-targeting strategy. Cancer-specific conventional chemotherapeutic agents have been used in combination with ER stressor agents and autophagy inhibition for six models of cancer: PC-3 (Figure 5A) and LNCaP (Figure 5B), primary human stromal prostate cancer cells (Figure 5F), MCF-7 and MDA-MB-231 breast cancer cells (Figures 5C, D) and MIA-PaCa2 pancreatic cancer cells (Figure 5E). Interestingly, in almost all conditions, the different autophagic inhibitors failed to significantly increase the chemotherapeutics apoptotic index. In contrast, ASP-8ADT used in combination with an autophagic inhibitor frequently increased chemotherapeutics cytotoxicity, regardless of their action mechanism, thus suggesting a unique way of priming cancer cells for several conventional cancer therapies. In the case of stromal prostate cancer cells and mammary cancer cells MCF-7, ASP-8ADT with autophagy inhibition induced high levels of apoptosis even in the absence of chemotherapy (Figures 5C, F). Most of our combined therapies were due to a synergic effect of the treatment (example of the analysis can be found in Figures 5G, H and S5A-C). Only few conditions were simply additive effects (pancreatic cell lines for cisplatin and 5-FU, cisplatin, docetaxel on MDA-MB-231).

We also conducted a series of combined therapy experiments on nude mice using prostate and breast tumor xenografts. The proof of principle concerning G-202 is well-established *in vivo* (Denmeade et al., 2012) and in clinical trials, G-202 is injected intravenously. However, G-

202 efficiency is also associated with an antiangiogenic effect that may biased our results when used in *in vivo* experiments with chemotherapeutic agents such as taxotere (docetaxel). Thus, in our experiments, to achieve ER Ca^{2+} stress, tumors were injected intratumorally (i.t.) on a basis of six injections over two weeks with ASP-8ADT alone (1 μM /PBS), or in combination with CQ (50 μM /PBS). The results of these experiments revealed that animals treated with a combined therapy displayed significantly less tumor growth than animals treated with ASP-8ADT alone (Figure 6A) associated with increased cell death, as revealed by TUNEL staining (Figure 6B) and PARP cleavage (Figure 6C). We also assessed the ability of the combined therapy in priming cancer cells to a classical chemotherapeutic agent such as docetaxel or 5-Fluoro-Uracile (5-FU) on respectively prostate and breast xenografts. To reduce the number of mice used, accordingly to our strict ethical rules, we have focused our experiment on proving the efficiency of the combined therapy and consequently did not assess docetaxel or 5-FU alone (based on the literature and strong *in vitro* data). The combined therapy (ASP-8ADT/CQ) again significantly increased the capacity of docetaxel and 5-FU to reduce tumor growth and increase apoptosis index when compared to docetaxel or 5-FU and CQ alone (Figures 6D-H). Concerning breast xenografts, the inhibition of tumor growth in mice treated with a combined therapy is correlated with a drastic increase in the proportion of cells that underwent apoptosis, as revealed by TUNEL staining (Figure S6). We have used fresh human tumor samples from prostate in an *ex vivo* slices tumor assay to confirm the efficiency of autophagy inhibition on ASP-8ADT anti-tumor activity alone or combined with conventional chemotherapy. This *ex vivo* tumor assay is an excellent tool as it keeps tumor heterogeneity and allows the clinical response to anticancer drugs to be predicted (Majumder et al., 2015). In these experiments, human prostate tumors were treated with a complete panel of treatments. First, we confirmed the efficiency of autophagy inhibition on ASP-8ADT anti-tumor activity with an important increase in the proportion of cells undergoing apoptosis, as re-

vealed by TUNEL staining (Figure 6I). Second, the combined therapy (ASP-8ADT/CQ) significantly increased the capacity of docetaxel/CQ to induce apoptosis (Figure 6I). Altogether, these assays clearly showed that inhibition of autophagy rescues the anti-cancer activity of ASP-8ADT and that a combined therapy based on a Ca^{2+} stress and inhibition of autophagy increases chemosensitivity to docetaxel and 5-FU of prostate and breast cancer, respectively.

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Discussion

In a previous work, we uncovered an atypical mode to escape from cell-death in response to Ca^{2+} homeostasis perturbations (Dubois et al., 2016, 2013) but the precise molecular mechanisms were not investigated. Here we identified autophagy and mitochondrial fission as gatekeepers in the paradigm referred as the Ca^{2+} apoptosis link (Berridge et al., 2000). This dependency has never been identified before as a required step. Our most important finding is also that Ca^{2+} -induced MPTP opening cannot be longer considered as a potent apoptosis activator just by itself. Indeed, one of our most striking results is that while TG and its analogue ASP-8ADT induce similar ER, cytosolic and mitochondria Ca^{2+} dynamics leading to MPTP opening, apoptosis induced by ASP-8ADT is very modest (Figures 1, 2). We show that this “loss of function” is linked to its inability to block autophagy as it has been described for TG (Engedal et al., 2013; Ganley et al., 2011; Sætre et al., 2015), thereby revealing a direct and crucial role of autophagy in the Ca^{2+} apoptosis link (Figure 3). This feature may be supported by the fact that TG and ASP-8ADT, have similar but not identical chemical structure (Dubois et al., 2013). Originally it has been suggested that the inhibitory effect of TG on the autophagic flux, was independent of a functional ER stress response, but could be related to the activity of TG to inhibit SERCA and thus to influence downstream Ca^{2+} signaling pathways involved in the regulation of autophagy (Ganley et al., 2011). Our results are not in accordance with this possibility since ASP-8ADT induces both similar ER stress response and remodeling of Ca^{2+} homeostasis compared to TG (Figure 1). This implies that TG targets an unidentified molecular mechanism preferentially implicated in the closure of autophagosomes as it has been suggested in a previous study (Engedal et al., 2013). Nonetheless, we were able to restore the pro-apoptotic potency of ASP-8ADT by using pharmacological or genetic inhibition of autophagy.

We also demonstrated that both TG and ASP-8ADT induced potent mitochondrial Ca^{2+} overload that causes MPTP opening. However, we observed that mitochondria are still able to retain large amounts of Ca^{2+} and surprisingly, that the mitochondrial membrane potential could be preserved. These findings seem to be in contradiction with the fact that when MPTP is open, both Ca^{2+} and proton gradients across the inner mitochondrial membrane are dissipated. In fact, we identified that a situation with totally depleted mitochondria from Ca^{2+} can occur only when mitochondrial potential is totally collapsed by using a protonophore such as FCCP (Figure 4). Indeed, we observed that TG induced only partial mitochondrial depolarization compared to FCCP and we suggest that this feature could explain the retention of Ca^{2+} . In fact, our results are in accordance with some studies in that the process of MPTP opening at the cellular level does not obey to all-or-none law as it has been suggested in other models (Dumas et al., 2009). Concerning ASP-8ADT, the mitochondrial membrane potential was virtually not affected despite the opening of MPTP suggesting that other mechanisms can potentially play a crucial role for maintaining mitochondrial membrane potential. In particular, we identified autophagy and mitochondrial dynamics as two crucial steps involved in the maintain of mitochondrial potential (Figures 3, 4). Interestingly, we demonstrated that the pro-survival action of autophagy or mitochondrial dynamics could be used sequentially by the cells to try to overcome the stress mediated by mitochondrial Ca^{2+} overload (Figures 4). Indeed, in the case of autophagy impairment, cells switch from the pro-survival autophagy pathway to a DRP-1 mediated mitochondrial dynamics to try to overcome the stress mediated by mitochondrial Ca^{2+} overload, highlighting the role of DRP1 for maintaining mitochondrial potential as observed in other study (Choi et al., 2013). In this context, our results clearly indicated that the pharmacological or genetic impairment of DRP-1 could be used to restore the Ca^{2+} apoptosis link.

Thus, our study highlights the central role of autophagy and mitochondrial dynamics as survival mechanisms ensuring the integrity of mitochondrial membrane potential during mitochondrial Ca^{2+} overload stress. For this reason, the targeting of autophagy or mitochondrial dynamics could be used to promote apoptosis in cancer cells subjected to mitochondrial Ca^{2+} overload.

Our preclinical data confirmed that the use of Chloroquine or Rab7 GTPase inhibitor by inhibiting autophagy, increase chemotherapy efficiency in several cancer model *in vitro*. As a proof of concept, we confirmed the potential translational significance of our combined strategy with conventional drugs used in clinic or in clinical evaluation (i.e. ASP-8ADT). For this, we used both classical *in vivo* approach (xenograft mouse model) and an *ex-vivo* strategy with human clinical specimens of prostate and breast cancer (Figures 6, S6). This *ex-vivo* strategy allows the prediction of the clinical response to anticancer drugs by maintaining tumor heterogeneity (Majumder et al., 2015). In the case of prostate cancer treatment, we have used docetaxel which was the first chemotherapeutic agent to increase survival time in patients with androgen-resistant prostate cancer. For breast cancer we have evaluated the conventional treatment 5-FU. The ASP-8ADT compound used in our study to induce sustained mitochondrial perturbations corresponds to the active ingredient of the Mipsagargin pro-drug (G-202) (Denmeade et al., 2012) currently under evaluation as potential targeted therapy for prostate cancer, gliomas, clear cell renal cancer, or hepato-carcinomas. In a clinical study on patients with advanced solid tumors receiving Mipsagargin, authors did not observed a clinical response, but a prolonged disease stabilization was observed in a subset of patients (Mahalingam et al., 2016). In this context, our findings may benefit to patients by improving the clinical outcome associated with G-202 treatment. Indeed, our fundamental findings redefine the molecular pathways underlying the anti-tumor activity of ASP-8ADT (G-202) as we clearly demonstrated that concomitant inhibition of autophagy is required. In agreement with

these findings, we proposed two new combined anticancer therapies (1) G-202 combined with CQ (2) G-202 combined with CQ and a standard chemotherapy. Concerning the combination with a standard chemotherapy, we clearly demonstrated that the combination of ASP-8ADT and docetaxel in the background of autophagy inhibition shown synergistic interactions (Figure 6). It is also well known that mitochondrial priming mediated by BCL-2 family proteins correlates with clinical response to cytotoxic chemotherapy (Ni Chonghaile et al., 2011). Indeed, permeabilization of the OMM is considered to be the decisive event in the onset of cell death (Kroemer et al., 2007) and is subject to a tight control by pro- and anti-apoptotic members of the BCL-2 protein family (Czabotar et al., 2014). Interestingly, the mechanism identified here for the sensitivity and the resistance to cancer therapies seems to be a BCL-2 independent mechanism and thus, it may represent an innovative therapeutic approach. Importantly, all the compounds used in our proposed combination therapies have been approved by the FDA. All compounds used in our combined therapies have also distinct mechanisms of anti-tumor activity ruling out a possible competitive impairment and both CQ and G-202 have already demonstrated an acceptable tolerability (Manic et al., 2014). Such combined therapy using a targeted anticancer agent such as Mipsagargin in combination with a standard chemotherapy and inhibition of autophagy is a realistic approach. Recently Inspyr Therapeutics (G-202) have initiated a pre-clinical study evaluating the potential of G-202 in combination with Nexavar, using liver tumor models that express PSMA (Prostate Specific Membrane Antigen), the target of G-202. Moreover, numerous clinical studies also evaluate combined therapies including 2, 3 or 4 compounds. In particular, the combination of oxaliplatin, irinotecan, and 5-fluorouracil (5-FU) (FOLFOXIRI) in association with bevacizumab demonstrated in a phase III trial to improve outcome and became a new standard regimen for initial therapy of metastatic colorectal cancer (Loupakis et al., 2014).

In conclusion, these findings challenge a crucial paradigm in cell death revealing a novel and promising priming strategy to improve clinical outcomes for patients with solid cancers.

Limitations of the Study

Based on the results derived from pharmacological induction of mitochondrial Ca^{2+} overload in cultured cells and xenograft mouse models, we were able to demonstrate that, by inhibiting the autophagy pathway, mitochondrial Ca^{2+} status can modulate chemosensitivity. However, the conclusions would be strengthened if the precise molecular mechanism underlying autophagy inhibition and mediated by TG was identified. Moreover, the physiological and physiopathological aspects of this new interplay between Ca^{2+} signaling and autophagy in the regulation of mitochondrial cell fate should be confirmed in the context of pathologies associated with mitochondrial disorders (neurodegenerative and cardiovascular diseases, diabetes or myopathies).

Resource Availability

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Materials Availability

Further information required to interpret, replicate or build upon the methods or findings reported in the manuscript is available from the corresponding author upon request (Fabien.vanden-abeele@inserm.fr).

Data and Code Availability

There is no dataset and/or code associated with the paper.

Methods

Acknowledgments

We acknowledge Prof R. Tsien for generously providing 4mtTM3cpv Cameleon construct, and Prof J. Vuust Møller & Prof B. Christensen for providing thapsigargin analogues. Graphical Abstract was adapted from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Generic License. <http://smart.servier.com/>.

This work was supported by a grant from Comité Septentrion de la Ligue contre le cancer to FV and La Fondation de France to CD.

Authors Contributions

CD designed research studies, performed most of the experiments, conceived the experimental designs, analyzed the data and drafted the manuscript. AK, LN and GB performed experiments and analyzed the data. EV, FV and RT performed experiments. MR provided primary human stromal prostate cancer cells. DT participated to *in vivo* and *ex-vivo* experiments. JB provided primary human prostate cancer cells. NP provided advices. FV directed the study, designed research studies, performed the experiments, conceived the experimental designs, analyzed the data and drafted the manuscript.

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Declaration of Interests

The authors declare that they have no competing interests.

Ethics declarations

The present study was approved by Ethics Committee of GHICL Hospital, Hospital St-Philibert, Lomme, France.

Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with the local animal ethical committee in the animal house (C59-00913; protocol CEEA 202012) of the University of Sciences and Technologies of Lille, under the supervision of Dr. Lehen'kyi (59-009270).

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<https://doi.org/10.1016/j.neuropharm.2014.07.002>

Figure Legends

Figure 1: Redefining the Ca²⁺-Apoptosis Link. (A) Representative measurements of SOCE activated by SERCA pump inhibitors (TG; TG analogues: ASP-8ADT, LEU-8ADT, EPO-8ADT; CPA), as indicated by [Ca²⁺]_c elevation in LNCaP cells. (B) Quantification of [Ca²⁺]_c in LNCaP cells subjected or not to cytosolic Ca²⁺ overload 18 hr after SERCA pump inhibitor treatment. (C) Representative recordings of FCCP induced [Ca²⁺]_c elevation in LNCaP cells subjected or not to mitochondria Ca²⁺ overload 18 hr after SERCA pump inhibitors treatment. (D) Quantification of the results presented in (C). (E) Quantification of apoptotic cells, as determined by Hoechst staining, 48 hr after treatment with the control or the indicated drug (TG, 1 μM; ASP-8ADT, 1 μM; EPO-8ADT, 1 μM; LEU-8ADT, 1 μM and CPA, 10 μM). (F) Representative Western blot of the apoptosis marker protein PARP in LNCaP cells after 24 hr treatment with control or the indicated drug as in (E). Data are representative of 3 independent experiments. (G) TUNEL staining of LNCaP cells 18 hr after treatment. (H, I, J) Quantification of apoptotic cells, as determined by Hoechst staining, 48 hr after treatment with control or the indicated drug in PC-3 (prostate), MDA-MB-231 (breast) and MIA-PaCa2 (pancreatic) cancer cells. (K) Images show photon intensity (top) and phase lifetime (down), τ(φ), of Cerulean fluorescent protein encoded in the mitochondria-target Cameleon biosensor, 4mtTM3cpv, expressed in LNCaP cells for 36 hr. Cells were incubated with either TG or ASP-8ADT compounds for 24 hr prior to performed TD-FLIM experiment. Experiments were performed twice independently, and 15 cells were analyzed per experiment. (L) Quantification of ROS production using flow cytometry after 18 hr of treatment with TG (1 μM), ASP-8ADT (1 μM). Pyocyanin (100 μM / 30 min) is used as a ROS inducer. Oxidative Stress Detection Reagent (green fluorescent) for total ROS detection is used (ENZO LIFE SCIENCES). Experiments performed in triplicate. Bars represent mean ± SEM.

*P < 0.05, Student's t test, as compared with corresponding control.

Figure 2: Ca²⁺ homeostasis disruption and MPTP opening. (A) MPTP opening was assessed directly by the calcein/Co²⁺ method and calcein fluorescence quenching was imaged after 18 hr of treatment with TG (1 μM) or ASP-8ADT (1 μM). (B) Quantification of the results presented in (A). (C) Effect of CsA and IM on MPTP opening assessed by calcein/Co²⁺ method after 18 hr of treatment with TG (1 μM) or ASP-8ADT (1 μM). (CsA; 0,5 μM). (IM; 1 μM). (D) Quantification of mitochondrial Ca²⁺ content in LNCaP cells subjected or not to cytosolic Ca²⁺ overload, 18 hr after SERCA pump inhibition by TG or ASP-8ADT. CsA (0,5 μM) or FCCP (1 μM) pre-treatment (19 hr. 1 hr before SERCA pump inhibition). (E) The subcellular distribution of cyt *c* after 18 hr of treatment in LNCaP cells, visualized using an anti-cyt *c* antibody (green fluorescence) using a confocal microscope. Arrows indicate apoptotic bodies or nuclei condensation (cell death) visualized by DAPI staining (blue). TG (1 μM), TG analogues (1 μM) and CPA (10 μM). (F) Quantification of cyt *c* release (% of cells). (G) Representative confocal images of LNCaP cells loaded with JC-1 dye after 18 hr of treatment with TG (1 μM) or ASP-8ADT (1 μM). (H) Quantification of the fluorescence ratio (red fluorescence due to JC-1-aggregate/green fluorescence due to JC-1 monomers) from experiments presented in (G). Experiments performed in triplicate. Bars represent mean ± SEM. *P < 0.05, Student's t test.

Figure 3: Differential effects of thapsigargin analogues on autophagy. (A) Representative confocal images of LNCaP cells transiently transfected with a tandem-tagged GFP-mCherry-LC3 probe and treated as indicated for 2 hr with TG (1 μM) or ASP-8ADT (1 μM) alone or in combination with chloroquine (CQ, 25 μM). (B) Quantification of autophagic flux using the ratio (mCherry/GFP positives) / (mCherry positives only). (C) Size of acid vesicles of the

results presented in (A). (D) Representative Western blot of the autophagy markers protein P62 (upper panel) and LC3 I & II (middle panel) in LNCaP cells after treatment with control or the indicated drug with same concentration as used in (A). Rab7 inhibitory peptide (100 nM). (E) Quantification of LC3 proteins using the ratio (LC3II)/(LC3I+LC3II) protein levels. Values represent the relative density of bands compared to the control condition, which will obviously have a relative density of 1. n=3. (F) Quantification of P62 protein levels. Values represent the relative density of bands compared to the control condition, which will obviously have a relative density of 1. n=3. (G) Electron microscopy images of LNCaP cells treated as indicated for 48 hr with TG (1 μ M) or ASP-8ADT (1 μ M). The dashed line box is a magnification of a region of interest showing incomplete autophagic structures (unclosed autophagosome: black arrows). Experiments performed in triplicate. Bars represent mean \pm SEM. *P < 0.05, Student's t test.

Figure 4: Inhibition of autophagy determines the pro-apoptotic potential of mitochondrial Ca²⁺ overload stimulus by allowing mitochondrial fission and membrane depolarization. (A, B) Quantification of apoptotic cells, as determined by Hoechst staining, 48 hr after treatment with control, ASP-8ADT alone or in combination with autophagy inhibitors: Rab7 inhibitory peptide (100 nM), Chloroquine (CQ, 25 μ M), Wortmanin (WT, 100 nM), and Bafilomycin A1 (Baf A1, 100 nM). Cells were previously subjected to the indicated siRNA-mediated silencing of ATG5 compared to control anti-luciferase siRNA (CTL) (24 hr). (C) Quantification of cells with depolarized mitochondria, as determined by the DIOC6(3) probe, 18 hr after treatment with the indicated drug compared to CTL. (D) Quantification of the fluorescence ratio from LNCaP, PC-3 and MCF-7 cells loaded with JC-1 dye after 18 hr of treatment. (E) Representative Western blot of the pro-apoptotic protein BAX and the anti-apoptotic protein Bcl-2 in LNCaP mitochondrial fraction after treatment.

n=3. (F) Representative confocal images of LNCaP and PC-3 cells transiently transfected with a GFP-mitochondria-probe and treated as indicated for 18 hr. White arrows indicate cells with mitochondrial fission. (G) Quantification of cells with mitochondrial fission from the results presented in (F). (H, I, J) Quantification of apoptotic cells, as determined by Hoechst staining, 48 hr after treatment with control or the indicated drug or with siRNA-mediated silencing of DRP-1 (24 hr, 20 nM). n=3. FCCP, 0,5 μ M. Experiments performed in triplicate. Bars represent mean \pm SEM. *P < 0.05, Student's t test.

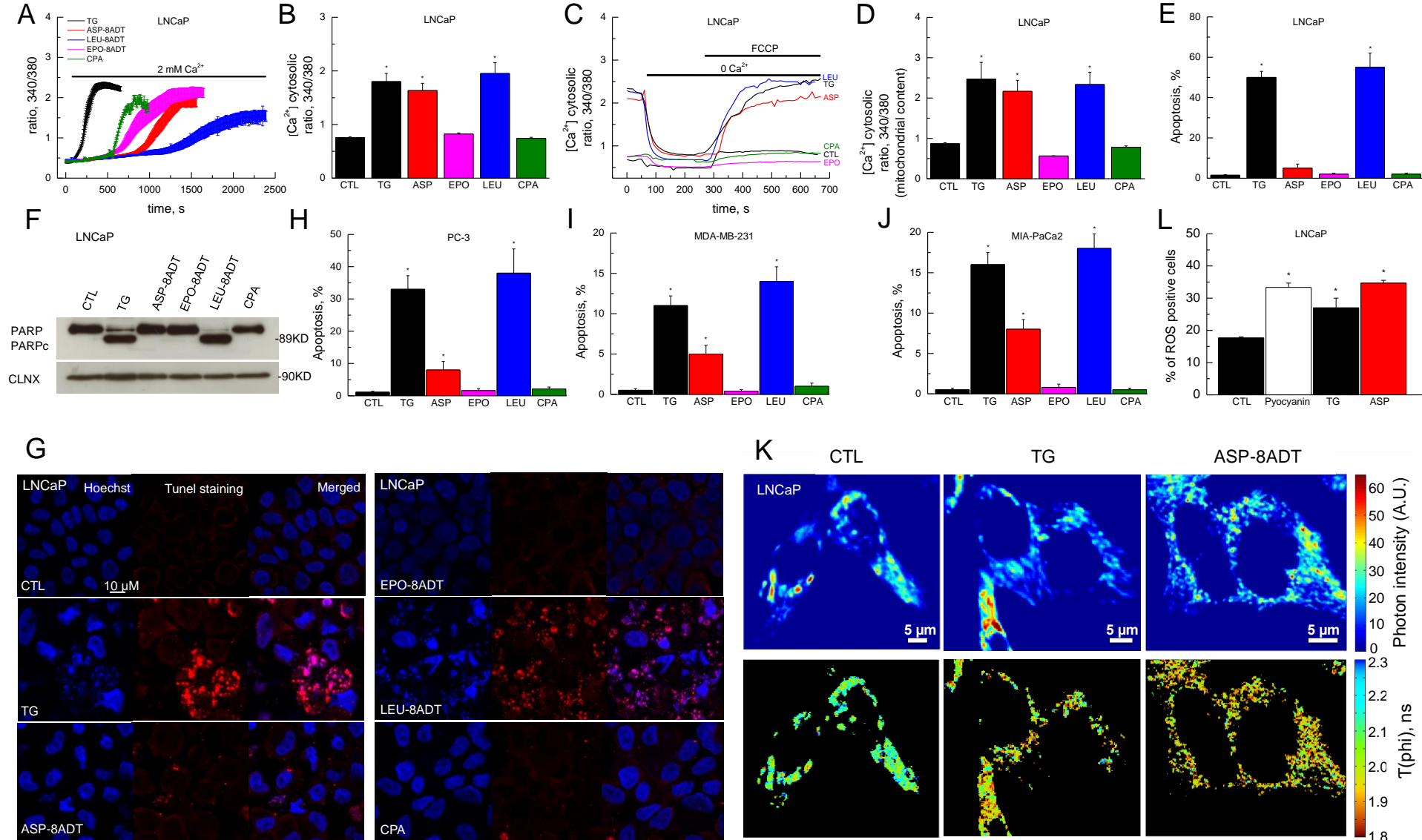
Figure 5: A co-targeting strategy based on mitochondrial Ca^{2+} overload and autophagy inhibition represents a crucial determinant of chemosensitivity.

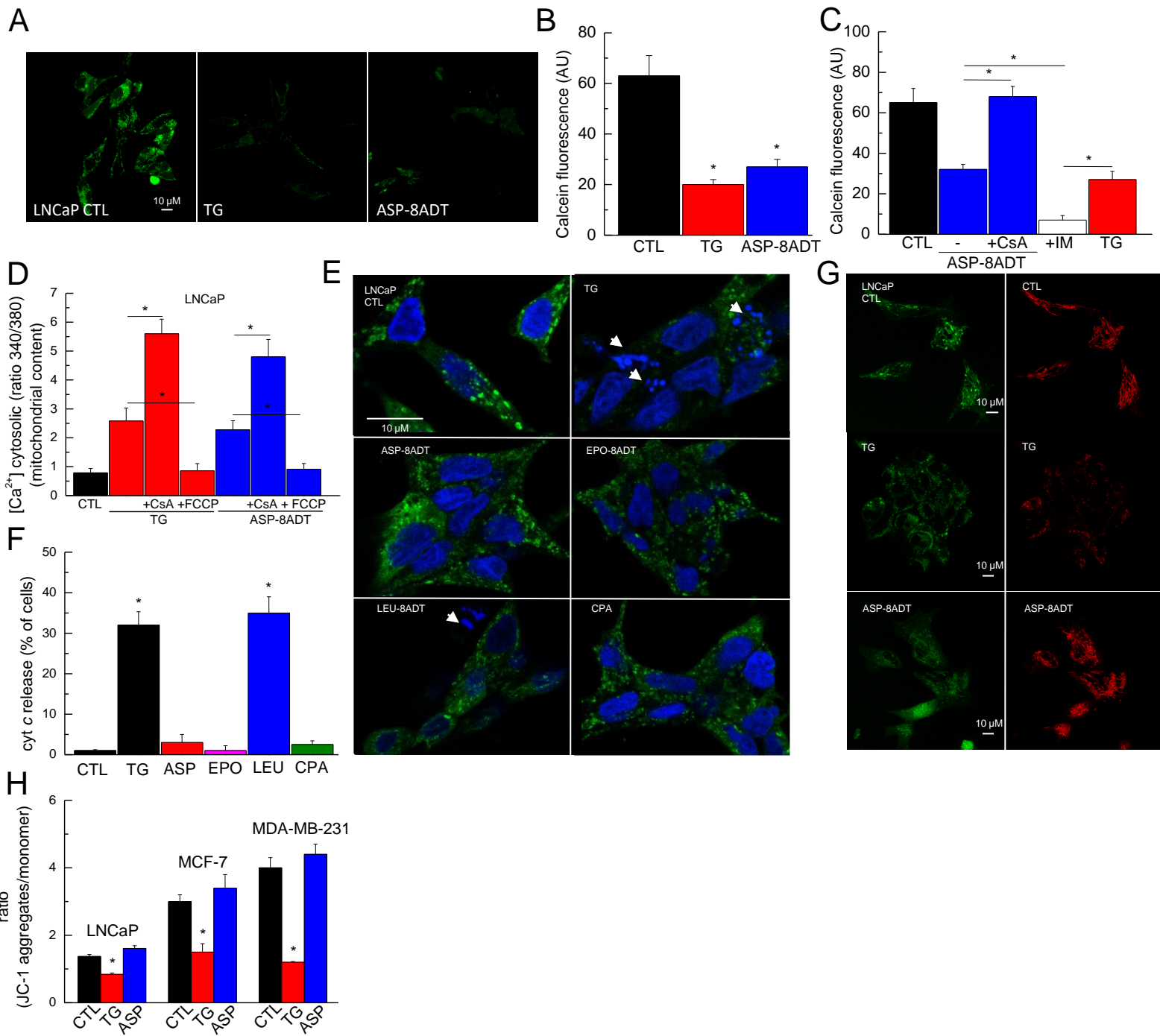
(A-F) Quantification of apoptotic cells, as determined by Hoechst staining, 48 hr after ASP-8ADT or TG-treatment with control or the indicated drugs in PC-3 cells (A), LNCaP (B), MCF-7 (C), MDA-MB-231 (D), MIA-PaCa2 (E), and stromal prostate cancer cells (F). n=3. (G-H) Isobologram and combination index (CI) analyses for evaluating ASP-8ADT/Docetaxel interactions in combination in LNCaP cells. Dose effect response curves were performed in the background of autophagy inhibition by the use of CQ and isobologram analyses were performed. Experiments performed in triplicate. Bars represent mean \pm SEM. *P < 0.05, Student's t test.

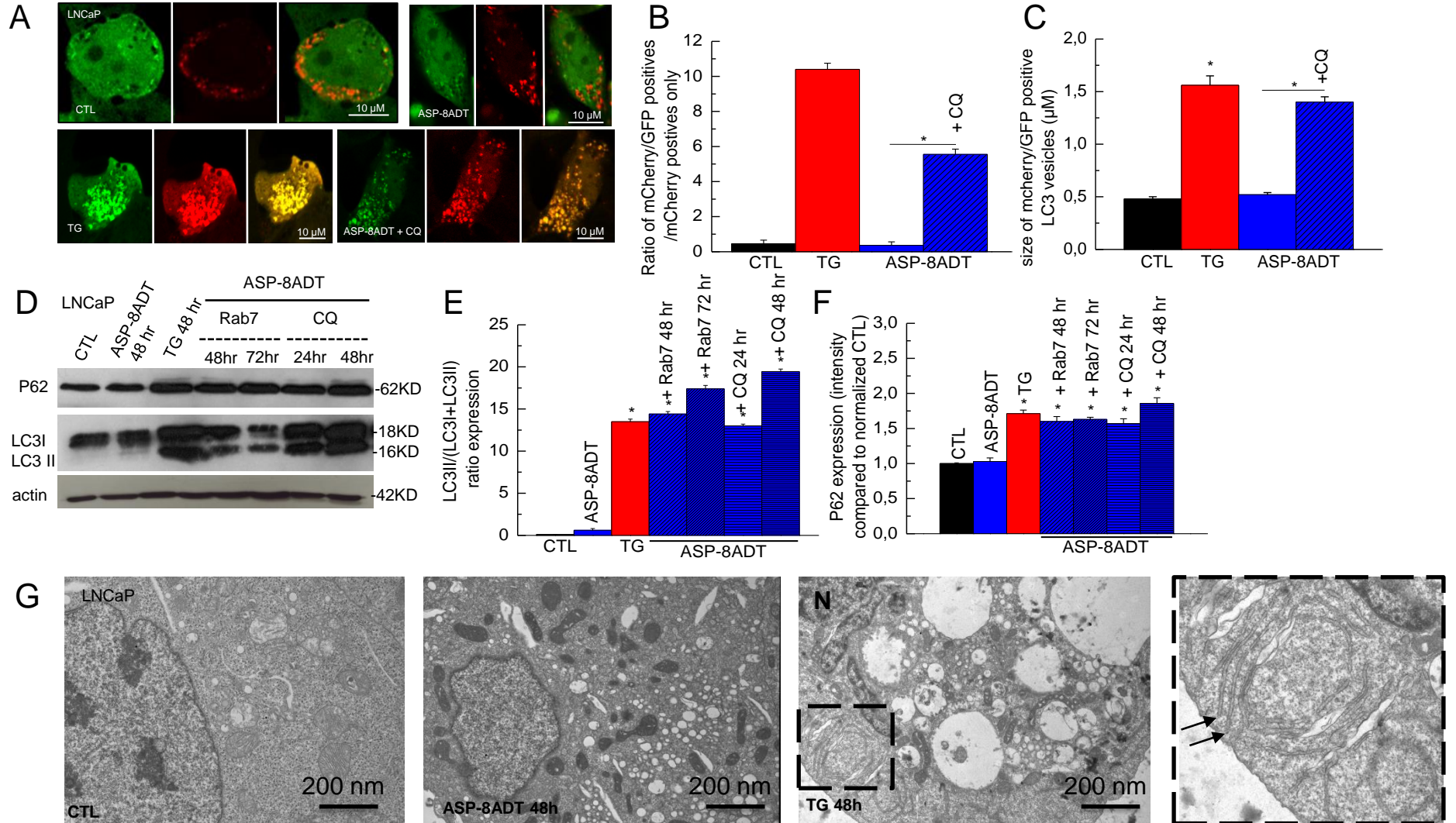
Figure 6: Priming cancer cells by a concomitant inhibition of autophagy and Ca^{2+} stress stimulation in mouse Xenograft tumor assays and in primary human tumor explants.

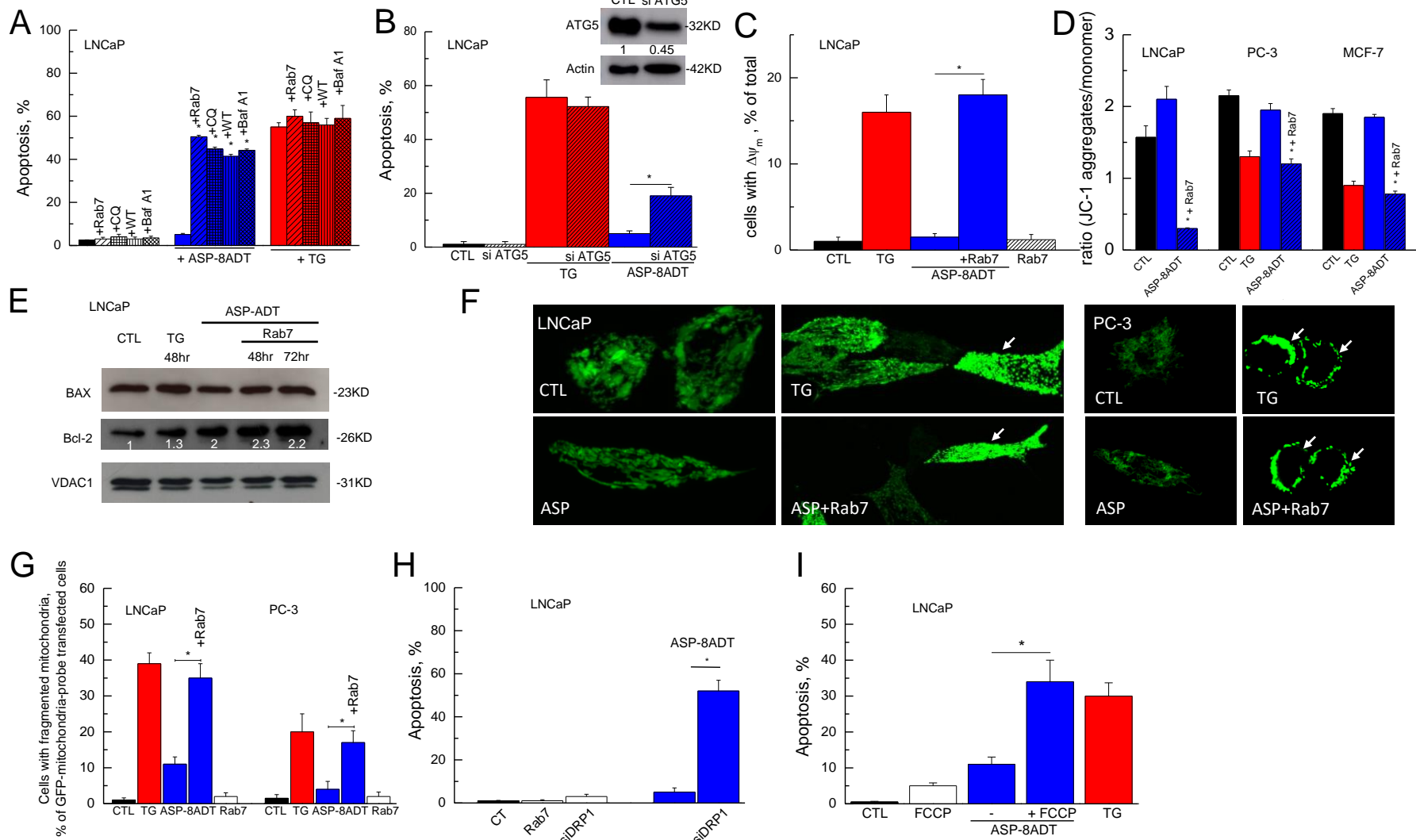
(A) The relative volume of PC-3 cell xenograft tumors over time in nude mice treated for 2 weeks with ASP-8ADT alone or in combination with CQ. (B) TUNEL staining obtained from tumor treated in (A). Colorimetric TUNEL Apoptosis Assay is used for detection of apoptosis. The 3'-OH end of the DNA strand breaks in apoptotic cells is labeled with biotinylated nucleotides using the enzyme TdT. Streptavidin conjugated horseradish peroxidase (HRP) is

then bound to the biotinylated nucleotides, and visualized using the peroxidase substrate, 3,3'-diaminobenzidine (DAB). The nuclei of apoptotic cells should be observed dark brown under light microscope. (C) A representative Western blot of the apoptosis marker protein PARP from tumors treated in (A). (D) The relative volume of PC-3 cell xenograft tumors over time in nude mice treated for 2 weeks with Docetaxel (10 mg/kg) alone or in combination with ASP-8ADT/CQ. (E) TUNEL staining obtained from tumor slices treated in (D). (F) A representative Western blot of the apoptosis marker protein PARP from tumors treated in (E). (G) Quantification of the relative tumor volume of PC-3 cell xenograft tumors treated for 2 weeks with Docetaxel/CQ or Docetaxel/CQ/ASP-8ADT. (H) The volume of MDA-MB-231 cell xenograft tumors over time in nude mice treated for 2 weeks with the indicated drugs. N=3 and n=5 per conditions for (A), (D) and (H). (I) Representative images of human prostate tumor sections labelled with Hoechst and TUNEL for apoptosis detection after *ex vivo* treatments with the indicated drugs (48 hr). n=4. Bars represent mean \pm SEM. *P < 0.05, Student's t test. The significance of the differences was analyzed using Student's t test at the end of the *in-vivo* experiments.









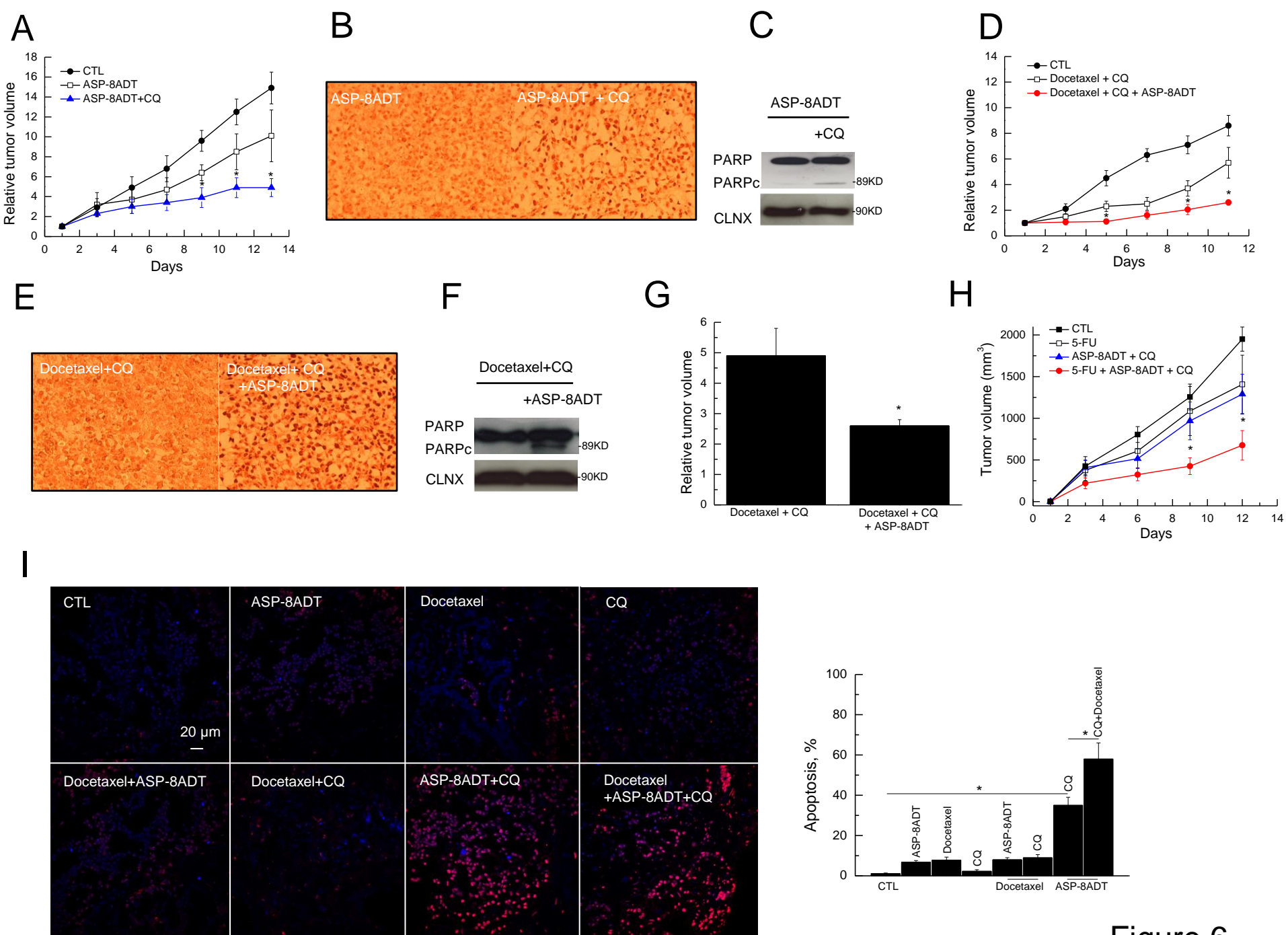


Figure 6

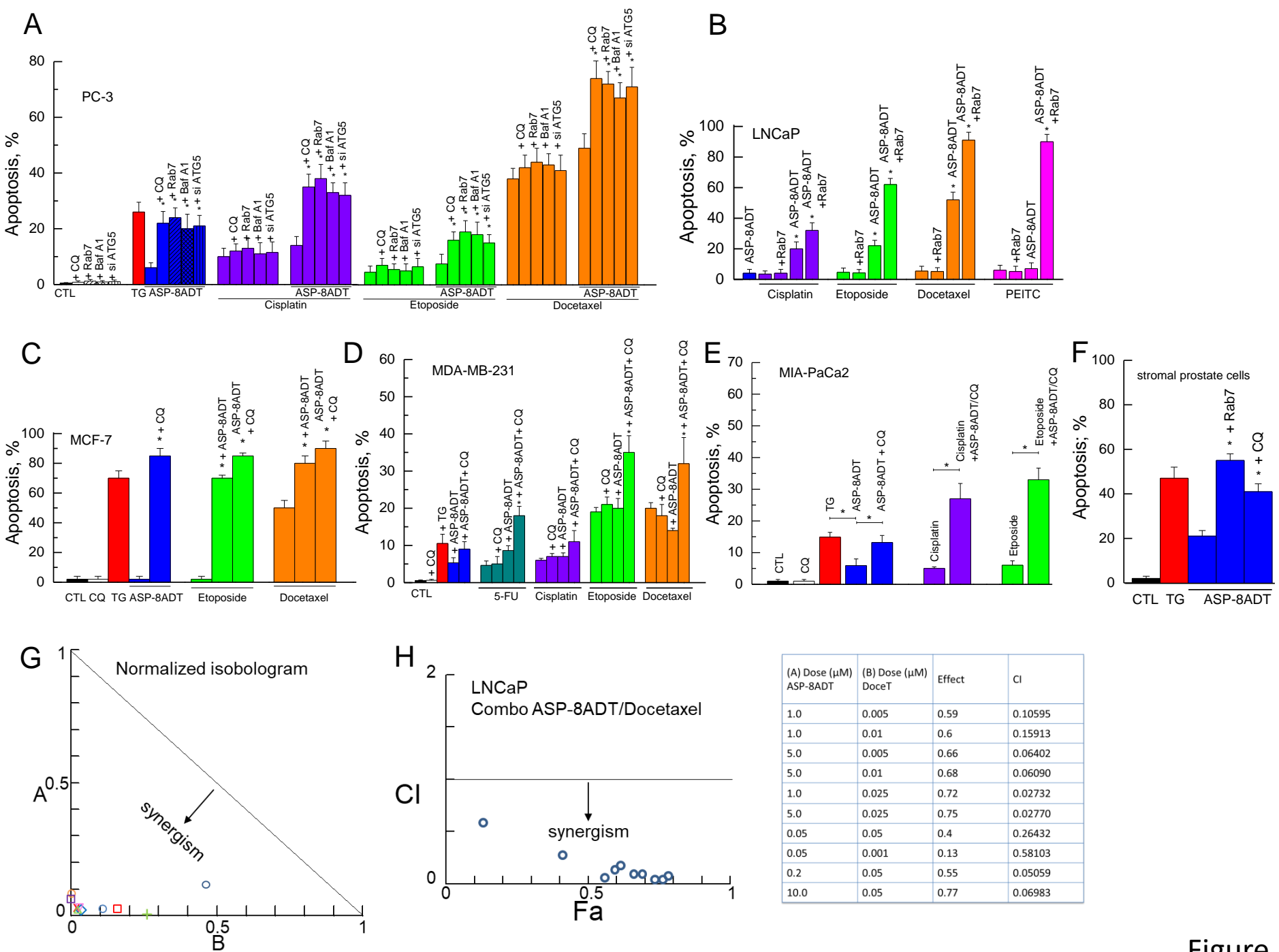


Figure 5

Highlights:

Mitochondrial Ca^{2+} overload stimulus alone is ineffective to induce cell-death

Autophagy is a gatekeeper of cell fate in response to Ca^{2+} homeostasis disruption

Mitochondrial Ca^{2+} overload is a determinant of autophagy as a chemosensitizer

Journal Pre-proof