An Early and Robust Activation of Caspases Heads Cells for a Regulated Form of Necrotic-like Cell Death*

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Background: Caspase activation triggers apoptotic cell death.
Results: Chelerythrine triggers necrotic-like cell death by an early and pronounced activation of caspases.
Conclusion: The rate and level of caspase activation dictate whether cells die displaying apoptotic or necrotic features.
Significance: Most antitumor drugs prompt concomitant apoptotic and necrotic morphological deaths; therefore understanding how the apoptosis-necrosis continuum occurs should shed light on anticancer treatment efficacy.

Apoptosis is triggered by the activation of caspases and characterized by chromatin condensation and nuclear fragmentation (type II nuclear morphology). Necrosis is depicted by a gain in cell volume (oncosis), swelling of organelles, plasma membrane leakage, and subsequent loss of intracellular contents. Although considered as different cell death entities, there is an overlap between apoptosis and necrosis. In this sense, mounting evidence suggests that both processes can be morphological expressions of a common biochemical network known as “apoptosis-necrosis continuum.” To gain insight into the events driving the apoptosis-necrosis continuum, apoptotically proficient cells were screened facing several apoptotic inducers for the absence of type II apoptotic nuclear morphologies. Chelerythrine was selected for further studies based on its cytotoxicity and the lack of apoptotic nuclear alterations. Chelerythrine triggered an early plasma membrane leakage without condensed chromatin aggregates. Ultrastructural analysis revealed that chelerythrine-mediated cytotoxicity was compatible with a necrotic-like type of cell death. Biochemically, chelerythrine induced the activation of caspases. Moreover, the inhibition of caspases prevented chelerythrine-triggered necrotic-like cell death. Compared with staurosporine, chelerythrine induced stronger caspase activation detectable at earlier times. After using a battery of chemicals, we found that high concentrations of thiolic antioxidants fully prevented chelerythrine-driven caspase activation and necrotic-like cell death. Lower amounts of thiolic antioxidants partially prevented chelerythrine-mediated cytotoxicity and allowed cells to display type II apoptotic nuclear morphology correlating with a delay in caspase-3 activation. Altogether, these data support that an early and pronounced activation of caspases can drive cells to undergo a form of necrotic-like regulated cell death.

Old, damaged, or superfluous cells from multicellular organisms undergo regulated cell death to preserve the correct homœostasis. Alterations in the intracellular lethal pathways can lead cells toward proliferative diseases, such as cancer. Accordingly, the identification and classification of the existing subroutines of cell death have drawn a great amount of attention (1). Research in the field has focused on apoptosis and necrosis and the identification of their biochemical and morphological differences. Apoptotic dying cells undergo cellular shrinkage and subsequent fragmentation into apoptotic bodies surrounded by plasma membrane, reducing the occurrence of proinflammatory responses (2, 3). Nuclear apoptotic changes involve a partial chromatin condensation around the nuclear envelope (type I apoptotic nuclear morphology) and/or fragmentation of the nuclei into highly packaged round masses of condensed chromatin (type II apoptotic nuclear morphology) with this last stage one of the most distinctive morphological characteristics of apoptosis (4, 5). One of the most studied biochemical hallmarks of apoptosis is the precise activation in both space and time of a family of cysteine proteases called caspases. Caspases are subdivided into initiator or instigator (caspase-8, -9, and -10) and effector or executioner (caspase-3, -6, and -7) pro-

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The abbreviations used are: BH3, Bcl-2 homology 3; ABT-737, 4-[4-[4-[2-(4-

Materials and Methods

Reagents—All chemicals were obtained from Sigma unless otherwise indicated. Thapsigargin, nocardazole, rotenone, and colchicine were purchased from Merck KGaA. The inhibitors of caspases Z-VAD(OMe)-fmk and q-VD-Oph were obtained from MP Biomedicals Europe (Illkirch, France). BH3 mimetics and PKC inhibitors were purchased from Selleckchem (Houston, Texas). The caspase substrate Ac-DEVD-afc was obtained from Calbiochem. Antibodies against caspase-3 (9662; 1:2000) and caspase-7 (9492; 1:2000) were purchased from Cell Signaling Technology (Beverly, MA). Antibody against α-fodrin (clone AA6) (MAB1622; 1:40,000) was obtained from Millipore Iberica S.A.U. (Madrid, Spain). Antibodies against caspase-9 (clone 5B4) (M054-3; 1:5000) and caspase-6 (clone 3E8) (M070-3; 1:2000) were from MBL International Corp. (Naka-ku Nagoya, Japan). Anti-caspase-2 (clone 11B4) (ALX-804-356; 1:5000) was purchased from Enzo Life Sciences (Lausen, Switzerland). Anti-lamin A/C (clone JOL2) (AB40567; 1:2000) was from Abcam (Cambridge, UK). Anti-p23 antibody (clone J13) (NB300-576; 1:10,000) was obtained from Novus Biological Europe, Inc. (Cambridge, UK). Horse-radish peroxidase-conjugated secondary antibodies against mouse IgG (A9044; 1:10,000), rabbit IgG (A0545; 1:20,000), and rat IgG (A9037; 1:20,000) were purchased from Sigma.

Cell Lines and Culture Procedures—Human neuroblastoma-derived SH-SY5Y, SK-N-AS, SK-N-SH, IMR-5, IMR-32, LAN-1, SK-N-BE(2), and SK-N-JD cell lines were routinely grown in 100-mm culture dishes (BD Falcon, Madrid, Spain) containing 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively) and 10% heat-inactivated fetal bovine serum (Invitrogen S.A.). Medium was routinely changed every 3 days. Cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air and 5% CO2.

For the different experiments, cells were grown at the adequate cell densities in culture dishes or multwell plates (BD Falcon) using the same culture conditions as described above.

Cell Treatments—The following cytotoxic drugs were used: staurosporine (1 μM), rotenone (100 μM), chelerythrine (40 μM for experiments in Fig. 1 and 10 μM for the rest), camptothecin (20 μM), etoposide (100 μM), oxaliplatin (50 μM), colchicine (10 μg/ml), nocardazole (50 μM), thapsigargin (50 μM), Ac-L-LnL-CHO (MG101; 50 μM), Ac-L-CCM-CHO (50 μM), MG132 (Ac-L-CCM-CHO; 25 μM), Z-LLnV-CHO (20 μM), and Z-LnN-CHO (MG115; 20 μM). The following chemical compounds were used in the presence or not of 10 μM chelerythrine: q-VD-Oph (20 μM), pepstatin A (100 μM), tosyl-arginyl-phenylalaninyl chloromethane (10 μM), 4-(2-aminoethyl)benzenesulfonyle fluoride hydrochloride (150 μM), Z-LLnL-CHO (20 μM), Z-LnN-CHO (20 μM), Z-VAD(OMe)-fmk (50 μM), Z-FA-fmk (100 μM), leupeptin (100 μM), Z-FL-COCHO (20 μM), Ac-LnLn-CHO (20 μM), Ac-L-CCM-CHO (20 μM), dithiothreitol (DTT; 2 mM), ethylenediaminetetraacetic acid (EDTA; 2 mM), methylated glutathione (Me-GSH; 5 mM), N-acetylcycteine (NAC; 5 mM), ascorbate (100 μM), α-tocopherol (1 mM), and Trolox (100 μM). Each one of these inhibitors was individually added to the culture medium 1 h before chelerythrine treatment.

Cell Viability Assays: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Reduction, Lactate Dehydrogenase Release, and Double Direct Staining of Nuclei with Propidium Iodide (PI) and Cell-permeable Bisbenzimide Hoechst 33342—3.5 × 104 cells/ml were seeded in 96-multwell plates to carry out different viability assays. The MTT survival assay was performed as established previously (16). The lactate dehydrogenase release assay was carried out by its enzymatic detection in the extracellular medium as described (17). Cell death was also determined by PI/Hoechst double staining (18). PI and cell-permeable Hoechst 33342 were added to the culture medium at a final concentration of 1 and 2 μg/ml, respectively. After 10 min of cold incubation, fluorescence images were obtained.
taken, and the percentage of cell death was determined by counting PI-positive cells over the total nuclei stained with Hoechst 33342. Images were obtained with a Nikon ECLIPSE TE2000-E microscope equipped with epifluorescence optics under UV illumination and a Hamamatsu ORCA-ER photographic camera.

Nuclear Morphology Analysis by Chromatin Staining with a Non-cell-permeable Bisbenzimide (Hoechst 33258)—3.5 × 10^5 cells/ml were seeded in 96-well multiplates, and we proceeded as established previously (19). The images were acquired through the Nikon ECLIPSE TE2000-E microscope as mentioned above. The percentages of apoptotic nuclei were obtained by counting bisbenzimide-stained nuclei with condensed chromatin and/or fragmented nuclei versus total bisbenzimide-stained nuclei.

Morphology Analysis by Transmission Electron Microscopy—5 × 10^5 cells/ml were seeded in 60-mm culture dishes. After treatment, cells were detached, pelleted at 500 × g for 5 min, and washed gently with PBS. Transmission electron microscopy was performed as reported previously (17).

Protein Extractions and Western Blotting—Cells were detached from 35-mm culture dishes, pelleted at 500 × g for 5 min, and washed once with PBS. Transmission electron microscopy was performed as reported previously (17).

Results

Chelyerythrine Is a Potent Cytotoxic Compound That Induces a Synchronic Necrotic-like Cell Death—We have previously focused on the molecular and biochemical mechanisms of non-canonical apoptotic cell death in human neuroblastoma-derived cell lines in response to different apoptotic insults. Indeed, we have described that human neuroblastoma-derived IMR-5 and SK-N-AS cells do not show complete apoptosis during caspase-dependent cell death (20–22). In contrast, human neuroblastoma-derived SH-SY5Y cells subjected to apoptotic triggers display nuclear alterations of canonical apoptotic cell death (type II nuclear morphology) (16, 21). According to this behavior, we selected SH-SY5Y cells to screen for cytotoxic drugs triggering the appearance of a homogenous population of non-apoptotic nuclei. We first evaluated the apoptotic behavior of SH-SY5Y cells facing a broad battery of different cytotoxic insults. Among them, we used staurosporine (broad kinase inhibitor); rotenone (inhibitor of mitochondrial electron transport chain complex I); chelyerythrine (protein kinase C inhibitor and BH3 mimetic); camtothecin and etoposide (topoisomerase I and II inhibitors, respectively); oxaliplatin (DNA cross-linker agent); colchicine and nocodazole (microtubule-destabilizing compounds); thapsigargin (sarcoendoplasmic reticulum calcium transport ATPase inhibitor); Ac-LLnL (or MG101) and Ac-LLM (calpain inhibitors); Z-FL (cathepsin S inhibitor) and MG132, Z-LLF, and Z-LLnV (or MG115) (proteasome inhibitors). The percentages of cell viability obtained by MTT assay after treating cells with the different compounds were as follows: staurosporine, 0.15 ± 0.10%; rotenone, 30.77 ± 2.16%; chelyerythrine, 0.08 ± 0.05%; camtothecin, 12.83 ± 2.26%; etoposide, 2.14 ± 0.48%; oxaliplatin, 8.37 ± 0.58%; colchicine, 27.90 ± 0.59%; nencodazole, 43.19 ± 2.23%; thapsigargin, 2.52 ± 1.21%; Ac-LLnL, 30.33 ± 0.96%; Ac-LLM, 49.03 ± 2.82%; Z-FL, 28.51 ± 1.36%; MG132, 40.57 ± 0.77%; Z-LLF, 22.98 ± 1.64%; and Z-LLnV, 35.03 ± 1.26% (Fig. 1C). Most of the compounds induced the appearance of both apoptotic and non-apoptotic nuclei. The percentage of apoptotic nuclear morphologies varied depending on the specific treatment as follows: staurosporine, 99.72 ± 0.20%; rotenone, 25.63 ± 1.97%; chelyerythrine, 2.35 ± 0.49%; camtothecin, 48.20 ± 4.69%; etoposide, 23.42 ± 2.40%; oxaliplatin, 54.83 ± 3.14%; colchicine, 16.43 ± 1.12%; nencodazole 43.19 ± 2.23%; thapsigargin, 27.59 ± 3.40%; Ac-LLnL, 41.92 ± 1.31%; Ac-LLM, 20.42 ± 2.35%; Z-FL, 45.13 ± 1.97%; MG132, 29.83 ± 3.02; Z-LLF, 39.98 ± 2.58; and Z-LLnV,
According to these results, staurosporine was the most effective compound at inducing apoptotic nuclear morphology (Fig. 1, A and B). Others, such as chelerythrine, camptothecin, etoposide, oxaliplatin, and thapsigargin, induced lower percentages of apoptotic nuclei despite the high cytotoxicity provoked (Fig. 1 C). Among them, we selected chelerythrine for further studies because it exhibited high cytotoxicity without inducing the appearance of apoptotic nuclei (Fig. 1, A and B). The nuclei from chelerythrine-treated SH-SYSY cells shrank without signs of apoptotic nuclear changes (chromatin condensation and/or nuclear fragmentation) (Fig. 1A). Most of the drugs used triggered a heterogene-
ous cell death except for staurosporine and chelerythrine, which induced a prominent cytotoxicity characterized by the presence of homogeneous populations of apoptotic and non-apoptotic nuclear morphologies, respectively. The same behavior was observed in different human neuroblastoma-derived cell lines. Although 40 μM chelerythrine induced loss of cell viability (SK-N-SH, 0.32 ± 0.10%; SK-N-AS, 1.93 ± 0.84%; SK-N-BE(2), 4.54 ± 0.98%; SK-N-JD, 1.97 ± 1.01%; IMR-5, 0.20 ± 0.16%; IMR-32, 1.34 ± 0.69%; and LAN-1, 3.30 ± 0.96%) (Fig. 1E), the alkaloid was unable to induce apoptotic nuclear changes in any of the cell lines used (Fig. 1D). This cytotoxic response was not shared by other BH3-mimetic compounds, such as ABT-737 (50 μM), gossypol (25 μM), TW-37 (50 μM), and obatoclax (5 μM), or PKC inhibitors, such as calphostin C (100 μM), sotrastaurin (50 μM), Go 6983 (25 μM), Go 6976 (100 μM), and GF109203X (12.5 μM), which after 24 h of treatment caused apoptotic cell death with type II nuclear morphology (data not shown). To avoid uncontrolled cell responses due to excessive concentrations of chelerythrine in the culture medium, we checked the minimal concentration inducing the maximal cytotoxicity. The presence of 10 μM chelerythrine in the culture medium was already highly cytotoxic (0.10 ± 0.05% survival by MTT assay, 78.33 ± 1.24% cytotoxicity by lactate dehydrogenase release assay, and 82.51 ± 0.69% PI-positive nuclei) because the percentages of cell death reached were comparable with those obtained after treating cells with 40 μM chelerythrine (0.08 ± 0.06% survival by MTT assay, 99.93 ± 0.99% cytotoxicity by lactate dehydrogenase release assay, and 97.86 ± 0.80% PI-positive nuclei) (Fig. 2A). Nevertheless, none of the different concentrations used induced the presence of apoptotic nuclei (Fig. 2A). According to these results and unless otherwise stated, we used 10 μM chelerythrine henceforth. We then compared the cell death profile and nuclear alterations induced by chelerythrine and staurosporine through a time course analysis. As shown in Fig. 2B, chelerythrine-induced cytotoxicity occurred at earlier times when compared with staurosporine. For example, whereas staurosporine needed 6 h to induce 50% cell viability loss (MTT assay), chelerythrine provoked the same biological effect after 30 min of treatment (Fig. 2B). In the same sense, although 30 min of chelerythrine proved to be enough to induce plasma membrane leakage (as shown by either lactate dehydrogenase release assay or PI staining), staurosporine-treated cells maintained their plasma membrane integrity up to 2 h (Fig. 2B). Annexin V/PI co-staining coupled with flow cytometry can distinguish live, early apoptotic, late apoptotic/early necrotic, and late necrotic cell populations (23, 24). As shown in Fig. 2C, chelerythrine did not raise annexin V-positive/PI-negative (early apoptotic) cells at different time points of treatment. Indeed, similarly to H2O2 but contrarily to staurosporine, chelerythrine increased the percentage of annexin V/PI-double stained (late apoptotic/early necrotic) cells from early times (between 1 and 3 h) of treatment (Fig. 2C). Finally, at longer times (6 h for chelerythrine or H2O2, and 24 h for staurosporine), injured cells partially shifted to an annexin V-negative/PI-positive (late necrosis) population irrespective of the treatment used (Fig. 2C). Therefore, chelerythrine-treated cells showed necrotic-like (annexin V-positive/PI-positive or annexin V-negative/PI-positive) features without exhibiting apoptotic-like (annexin V-positive/PI-negative) features.

Regarding the nuclear aspect, apoptotic nuclei (condensed or fragmented) were not detected at any concentration or time of chelerythrine treatment (Fig. 2, A and B). Indeed, electron microscopy revealed a nuclear coarse pattern of irregular non-interconnected chromatin masses scattered throughout the nucleus in chelerythrine-challenged cells just 1 h after treatment (Fig. 3, middle left image). These chromatin clumps were homogeneously distributed inside the nucleus and some were located beneath the nuclear envelope, which was preserved although it had a misshapen contour (Fig. 3, middle left image). After 6 h of chelerythrine treatment, the previously observed chromatin alterations were preserved even though the nuclear envelope was barely detected (data not shown). These ultrastructural nuclear alterations were in contrast with those observed in staurosporine-challenged cells, which displayed aggregates of highly condensed chromatin distributed under the nuclear envelope as dense rounded caps (Fig. 3, upper right image). Besides these nuclear changes, cells cultured in the presence of chelerythrine showed multiple breaks at the plasma membrane, and their cytoplasm became disorganized and vacuolized (Fig. 3, middle image). These alterations were more prominent after 6 h of treatment (data not shown). Moreover, chelerythrine-treated cells displayed a cytoplasm characterized by a more electron-lucent pattern compared with untreated or staurosporine-treated cells (Fig. 3). The ultrastructural traits observed in chelerythrine-injured cells were compatible with the cellular changes described for necrotic cell death (cell swelling, early loss of plasma membrane integrity, electron-lucent and vacuolized cytoplasm, cell rupture, and irregular chromatin destruction) (25–27). We then compared the ultrastructural changes from chelerythrine-treated cells with those induced by a necrotic stimulus such as hydrogen peroxide (28). After 24 h of treatment, the cytoplasm of hydrogen peroxide-exposed cells appeared completely disintegrated, and the chromatin was slightly packaged into irregular patches (Fig. 3, lower right image). This aspect was highly similar to what we observed after 24 h of chelerythrine treatment (Fig. 3, lower left image). Therefore, the chelerythrine-induced demise occurred without signs of apoptosis but shared necrotic morphological traits.

**Necrotic-like Cell Death Induced by Chelerythrine Is a Caspase-dependent Process**—Because a defect in the activation of caspases could explain the lack of apoptotic morphological traits, we analyzed the activation of these proteases after chelerythrine challenge. After 24 h, chelerythrine induced the cleavage of initiator caspase-2 and -9 to their respective active fragments, p32/p18 and p37/p35 (Fig. 4A, first panel). We also observed the processing of the executioner caspases, i.e. caspase-3, -6, and -7, into their p19/p17, p18, and p20 active fragments, respectively (Fig. 4A, second panel). Finally, we confirmed the activation of executioner caspases by assessing the cleavage of different specific substrates. As shown in Fig. 4A, chelerythrine triggered the cleavage of α-fodrin to the p120 fragment (caspase-3-mediated) (29), lamin C into the p37 fragment (caspase-6-mediated) (30, 31), and p23 co-chaperone to the p15 fragment (caspase-7-mediated) (32). These results highlighted the similarities between staurosporine and chel-
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erythrine regarding the activation of caspases and the ensuing cascade of proteolytic events (Fig. 4A). We then wanted to ascertain whether caspases were involved in the necrotic-like cell death induced by chelerythrine. For that, we treated cells with chelerythrine for 24 h in the presence or absence of the broad pan-caspase inhibitor q-VD-OPh. We also co-treated cells with staurosporine and q-VD-OPh as a positive control of caspase-dependent apoptosis (22). First, we corroborated the proper q-VD-OPh-mediated inhibition of caspases by Western blotting and a caspase activity assay. As shown in Fig. 4B, when q-VD-OPh was present in the culture medium, caspase-3 was not processed into its respective active fragments (p19 and p17). Accordingly, q-VD-OPh abolished DEVD-directed caspase activity triggered by chelerythrine (Fig. 4B, lower panel). Subsequently, we examined the cytotoxicity induced by chelerythrine in the presence of q-VD-OPh. By means of an
MTT reduction assay, we showed that chelerythrine-mediated loss of cell viability was partially prevented by q-VD-OPh (from 0.10 ± 0.08 to 46.49 ± 1.67%). Similar results were reported when cells were co-treated with q-VD-OPh and staurosporine (from 0.34 ± 0.21 to 40.33 ± 1.93%) (Fig. 4C, upper graph). We also analyzed the integrity of the plasma membrane by the lactate dehydrogenase release assay or by counting PI-positive nuclei (Fig. 4C, middle and lower graphs). As shown in Fig. 4C, the addition of q-VD-OPh to the culture medium of chelerythrine-treated cells reduced cytotoxicity from 76.92 ± 1.82 to 17.20 ± 1.51% (by lactate dehydrogenase release assay) and from 85.83 ± 2.05 to 4.98 ± 0.73% (PI-positive nuclei counting). Taking advantage of the double PI/Hoechst nuclear staining, we found that q-VD-OPh also prevented the mild nuclear changes observed after chelerythrine challenge (Fig. 4D).

Indeed, electron microscopy analysis confirmed that q-VD-OPh blocked the formation of the chromatin irregular masses observed in chelerythrine-treated cells (Fig. 4E). Additionally, chelerythrine/q-VD-OPh-co-treated cells recovered their cytoplasmic integrity. Hence, our results showed that cell death and...
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A

- - + STP
- + - + Che

Caspase-2

- Pro-caspase

p32

p18

Pro-caspase

p37

p35

120 kDa

15 kDa

Caspase-9

NB

15 kDa

B

- - + + + q-VD
- + - + + STP
- + - - - Che

Caspase-3

180 kDa

p19

p17

Pro-caspase

15 kDa

C

% Survival (MTT reduction)

- - q-VD

- + q-VD

% Cell death (LDH release)

- - q-VD

- + q-VD

Caspase activity (A.U.F.)

- Control

- Che

- STP

D

Control

e - - - - q-VD

q-VD-OPh

+ q-VD-OPh

Fodrin

p120

Lamin C

p37

p23

p15

180 kDa

15 kDa

NB

15 kDa

26 kDa

E

Che

STP

Che + q-VD

STP + q-VD
necrotic-like alterations induced by chelerythrine required the proper activation of caspases.

**Necrotic-like Cell Death Prompted by Chelerythrine Is Biochemically Characterized by an Early and Prominent Activation of Caspases**—Despite that both staurosporine- and chelerythrine-driven cytotoxicity relied on caspase activation, these alkaloids triggered apoptotic and necrotic-like phenotypes, respectively. To identify dissimilarities in the activation of these enzymes, we performed a time course analysis of caspase activation in both chelerythrine- and staurosporine-treated cells. By Western blotting, we observed that both drugs induced the processing of fodrin, lamin C, and p23 co-chaperone to p120, p37, and p15 fragments, respectively (Fig. 5A). However, these fragments were already evidenced after 2 h of chelerythrine treatment, whereas they were not detected until 4 h of staurosporine treatment. Similarly, a DEVD-directed caspase-like activity assay proved that after 2 h chelerythrine-injured cells displayed 8 times higher caspase activity than staurosporine-treated cells (Fig. 5A, lower graph). We then wanted to identify the earliest time point, prior to 2 h, at which chelerythrine induced the activation of caspases. The p120 and p15 fragments from fodrin and p23 co-chaperone processing, respectively, were already detected after 30 min, being fully evident at 1 h of chelerythrine treatment (Fig. 5B). Accordingly, the enzymatic assay revealed a marked increase in caspase activation at 30 min and a maximal activity 1 h after the treatment. In contrast, staurosporine barely induced the activation of caspases for the first 2 h of treatment (Fig. 5B). When looking at caspase-3, the main executioner protease involved in the apoptotic hallmarks, we observed an early activation in chelerythrine-injured cells compared with staurosporine-treated cells. Indeed, after 1 h of chelerythrine, cells displayed a robust processing of caspase-3 into its p19/p17 active fragments that was not observed in staurosporine-injured cells (Fig. 5C). Based on these results, we explored whether higher concentrations of staurosporine could reproduce the early processing of caspase-3 driven by chelerythrine. As shown in Fig. 5D, at 1 h of treatment, staurosporine was unable to induce the processing of caspase-3 into its active p19/p17 fragments even at very high concentrations (64 μM). Therefore, our results indicated that the early activation of caspases was a biochemical peculiarity of chelerythrine not shared by staurosporine, suggesting that the early activation of caspases could be a key event orchestrating necrotic-like cell death.

**Caspase-dependent Necrotic-like Cell Death Induced by Chelerythrine Is Prompted by Thiolic Oxidative Stress**—Next, we wanted to identify the upstream key event provoking the early activation of caspases triggered by chelerythrine. In addition to q-VD-OPh, we used a broad battery of inhibitors, including pepstatin A (aspartic protease inhibitor, such as cathepsin D); tosyl-L-phenylalanyl chloromethane (inhibitor of α-chymotrypsin-like and some non-caspase protease teases); 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (broad hydrophilic PMSF-like serine protease inhibitor); Z-LLF and Z-LLnV (or MG115) (proteasome inhibitors); Z-VAD (broad caspase inhibitor with additional ability to interfere with calpain and cathepsin activities); Z-FA (inhibitor of cysteine proteases, such as cathepsins B, L, and S); leupeptin (inhibitor of cysteine proteases, such as calpains, papain, and cathepsins B, H, and L, and some serine proteases, such as plasmin, kallikrein, and trypsin); Z-FL (cathepsin S inhibitor); Ac-LLnL (or MG101) and Ac-LLM (calpain inhibitors); DTT, MeGSH, and NAC (thiolic antioxidant agents); and ascorbate, α-tocopherol, and Trolox (antioxidant compounds with a major role in the prevention of lipid peroxidation). We tested their ability to prevent cytotoxicity after 24 h of chelerythrine treatment by means of PI/Hoechst double staining. Among the inhibitors tested and similarly to q-VD-OPh, Z-VAD reduced chelerythrine-triggered cytotoxicity from 91.56 ± 2.43 to 2.25 ± 0.05%. These data reassured that chelerythrine induced a caspase-dependent cell death. Interestingly, all thiolic antioxidants used (DTT, Me-GSH, and NAC) fully prevented chelerythrine-induced cytotoxicity (from 91.56 ± 2.43 to 5.79 ± 0.88% (DTT), to 10.26 ± 2.09% (Me-GSH), and to 7.90 ± 2.80% (NAC)). In contrast, the other antioxidants tested displayed only a minor effect on chelerythrine-triggered cell death (Fig. 6A). Moreover, the nuclear morphological analysis corroborated that only caspase inhibitors and thiolic antioxidants prevented the nuclear alterations provoked by chelerythrine (data not shown). Thus, our results suggested a key role of thiol oxidative stress processes in chelerythrine-induced cytotoxicity. We then asked whether thiol oxidative stress was involved in the activation of caspases triggered by chelerythrine. For this purpose, we assessed by Western blotting the cleavage of caspase-3 in cells treated for 24 h with chelerythrine in the presence or absence of the above mentioned antioxidants. Chelerythrine-induced caspase-3 p19/p17 active fragments were not detected when thiolic antioxidants were added to the culture medium (Fig. 6B). Similarly, the DEVD-directed activity assay revealed that chelerythrine-mediated activation of caspases was inhibited by any of the above mentioned thiolic antioxidants (Fig. 6B). Altogether, our results showed that chelerythrine-mediated necrotic-like cell death required strong thiolic oxidative damage, which seemed an upstream pivotal event.
event to activate caspases in a very short period after cytotoxic insult.

Necrotic-like Cell Death Induced by Chelerythrine Can Be Shifted to Apoptosis by the Fine-tuning of the Thiol Oxidative Intracellular Status—Taking into account the results obtained, we established that necrotic-like cell death induced by chelerythrine depended on the intracellular oxidative stress and subsequently on the activation of caspases. Because caspases played a key role in chelerythrine-triggered necrotic-like cytotoxicity, we wondered whether a gentle modulation of the thiol oxidative status could switch the cell death to one with apoptotic nuclear traits. We then evaluated cell viability and nuclear morphology after chelerythrine treatment in the presence of different concentrations of DTT. As shown in Fig. 7A, cells cultured in the presence of high concentrations of DTT (1 or 2 mM) were fully protected from chelerythrine-triggered cell death. Moreover, 1 mM DTT prevented the mild nuclear alterations induced by chelerythrine (Fig. 7B). At the other end, the lowest concentrations of DTT used (from 0.01 to 0.3 mM) did not alter chelerythrine-mediated cytotoxicity (Fig. 7A). Taking 0.1 mM as a representative concentration, we observed that DTT did not prevent the nuclear alterations induced by chel-
erythrine (Fig. 7B). When we used intermediate concentrations of DTT (from 0.4 to 0.9 mM), we observed a partial reduction of the cytotoxicity induced by chelerythrine (Fig. 7A). To analyze the nuclear morphology under these conditions, we chose 0.5 mM as the representative concentration of DTT. Interestingly, 0.5 mM DTT allowed chelerythrine-treated cells to display classical type II apoptotic nuclear morphology (Fig. 7B). As shown before in Fig. 5, caspases were processed early in chelerythrine-injured necrotic-like cells compared with staurosporine-treated apoptotic cells. To ensure that the rate of caspase activation is the mandatory biochemical event determining apoptotic- or necrotic-like outcomes, we assessed the activation of caspase-3, the key executioner caspase allowing the apoptotic hallmarks, after chelerythrine and DTT co-treatment. High concentrations of DTT (1 mM), which preserves cell viability (Fig. 7A), fully prevented caspase-3 processing (Fig. 7C). However, the presence of intermediate concentrations of DTT (0.5 mM) in the culture medium, which allowed the presence of apoptotic-like nuclei (Fig. 7B), delayed chelerythrine-triggered activation of caspase-3 (Fig. 7C). Indeed, in chelerythrine/DTT-co-treated cells, the activation of caspase-3 was not detected at 1 h but after 6 h of treatment, similarly to that observed in staurosporine-injured apoptotic cells (Figs. 5C and 7C). Altogether, our results show that chelerythrine can trigger necrotic-like or apoptotic cytotoxic fates (both caspase-dependent) driven by particular intracellular thiol oxidative stress.

**Discussion**

Here, we report that chelerythrine is a highly cytotoxic compound that synchronously triggers cell death in different human neuroblastoma-derived cells. SH-SYSY cells treated with chelerythrine die without any apoptotic morphological traits by undergoing a fast cytoplasmic disintegration and loss of plasma membrane integrity. Chelerythrine-driven cell death features a suite of traits of canonical necrotic (oncotic) cell death. In this sense, ultrastructural analysis demonstrates that chelerythrine-injured cells show electron-lucent vacuolized cytoplasm, early loss of plasma membrane integrity, and irregular and circumscribed DNA patches, which are typical morphological changes described in necrotic dying cells (9, 25, 26, 33–35). Intriguingly, chelerythrine triggers a prominent and early activation of caspases, which is necessary for the necrotic-like cell death to occur. These findings prove that an early activation of caspases can lead to cell demise with morphological traits resembling those observed in classical necrotic cell death. Chelerythrine-mediated cell death is not prevented by the action of several chemical inhibitors of calpains, cathepsins, proteasome, or other non-caspase proteases. However, high

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**FIGURE 6.** Chelerythrine-mediated caspase-dependent cell death is regulated by thiolic antioxidants. A and B, SH-SYSY cells were left untreated (Control) or treated for 24 h with different inhibitors in the absence (−) or presence (+) of chelerythrine (Che) as described under “Cell Treatments.” A, cell death was analyzed by PI staining. Data shown are the mean ± S.E. (n = 3). B, the processing of caspase-3 to its active fragments (p19 and p17) was examined by Western blotting (left panel). Naphthol blue (NB) staining served as a loading control. Caspase activity was assessed by a DEVD-directed activity assay (right panel). Data are represented as arbitrary units of fluorescence (A.U.F.) and expressed as mean ± S.E. (n = 3). Error bars represent S.E.

TPCK, tosyl-L-phenylalanyl chloromethane; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride.
Chelerythrine Triggers Necrotic-like Cell Death via Caspases

Many insults have the capacity to induce apoptosis and necrosis when used at low and high concentrations, respectively (36, 37, 43). Alternatively, some chemicals trigger concomitant signs of necrotic and apoptotic cell death modalities (44). This fact was evidenced in our initial drug screening whereby a series of chemical compounds was selected according to their ability to trigger a homogenous cytotoxic phenotype. All the tested drugs, with the exception of staurosporine and chelerythrine, trigger mixed subroutines of cell death as proving the same trigger is able to induce different types of cell demise (42). Our data support the notion that the apoptotic-necrotic continuum could rely on particular environmental cues, such as the thiol oxidative status of the cell.

The switch of apoptotic to necrotic paradigms of cell death has been profusely addressed (13–15, 36–39). In this regard, reducing the content of ATP or glutathione is a reported strategy to convert apoptotic to necrotic cell death (13, 15, 38, 39). Conversely, only a scarce number of publications have demonstrated that necrosis can be transformed into an apoptotic cell death; among them, we highlight the findings on prothymosin-α1 as a molecular switch of necrotic to apoptotic cell death (40, 41). Moreover, no reports have approached the possibility of turning a caspase-dependent necrotic-like cell death into an apoptotic-like process of cell demise. In this context, we prove that the fine-tuning of thiol oxidative processes is a successful strategy to modulate the shape of chelerythrine-mediated cell death by promoting a proper (delayed on time) activation of caspases. Indeed, we demonstrate for the first time that modulating the intracellular thiol oxidative environment by co-culturing cells with DTT switches chelerythrine-elicited necrotic-like cell death into an apoptotic-like process. This fact is of capital relevance because most antitumor drugs used in the clinics prompt a continuum of apoptotic and necrotic morphological deaths, thus proving the same trigger is able to induce different types of cell demise (42). Our data support the notion that the apoptotic-necrotic continuum could rely on particular environmental cues, such as the thiol oxidative status of the cell.

FIGURE 7. A delay in chelerythrine-triggered activation of caspase-3 prompted by intermediate concentrations of DTT allows cells to display apoptotic-like nuclear alterations. A, SH-SY5Y cells were left untreated (Control) or treated for 6 h with 2 mM DTT or 10 μM chelerythrine (Che) in the absence or presence of the indicated concentrations of DTT. Cell death was measured by PI staining. Data are represented as the mean ± S.E. (n = 3). B and C, cells were treated with 1 mM DTT, 10 μM chelerythrine, or a combination of 10 μM chelerythrine and different concentrations of DTT (1, 0.5, and 0.1 mM). B, after 24 h, nuclear morphology was assessed by nuclei staining with Hoechst. The insets are higher magnifications of the cells framed in the images. The scale bar indicates 50 μm. More than 1,000 nuclei from each condition were counted and scored as apoptotic (condensed or fragmented) or non-apoptotic nuclei. Graphs represent the means ± S.E. (n = 3). C, after the indicated times, cells were collected, and protein extracts were obtained. Caspase-3 cleavage into its p19/p17 active fragments was assessed by Western blotting. Naphthol blue (NB) staining served as a loading control. Note that in the presence of 0.5 mM DTT chelerythrine does not promote caspase-3 cleavage at 1 h but after 6 h of treatment, similar to the pattern observed in cells challenged with staurosporine (Fig. 5C). Error bars represent S.E.
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In the early increase of intracellular ROS elicited by chelerythrine parallels the activation of caspases. Furthermore, the mere attenuation of thiolic oxidative injury, which delays chelerythrine-driven caspase-3 activation, is sufficient to revert from the necrotic-like to apoptotic-like mode of cell demise.

Overall, our findings have uncovered an intriguing form of regulated necrosis, which relies on the activation of caspases in response to an intracellular thiolic oxidative stress. In the same line, a limited amount of work has intended to address the molecular pathways involved in different modes of caspase-dependent regulated necrosis. These studies identify several key factors for caspase-dependent necrotic-like cell death, such as the acidic pH (59) and the developmental stage of a specific cell type (52). However, none of those studies address how caspases can elicit necrotic-like cell death. Our findings establish that thiolic oxidative stress is another element to consider in caspase-dependent necrotic-like cell death and more importantly that the rate and level of caspase activity seem to be determinants to promote cell death with apoptotic-like or necrotic-like morphological traits. Notably, most of those elements, including the oxidative status, are naturally found in the tumor context, and thus they could influence the final mode of cell death elicited by a chemotherapeutic drug. In this sense, the molecular pathways leading to different subroutines of cell death are not trivial issues for anticancer drug applicability. On the contrary, they are central events determining the potential therapeutic and side effects of a drug. Malignant cells are often characterized by a lower extracellular pH (59) and a higher intrinsic oxidative stress than healthy cells (60, 61). In addition, escalated generation of ROS promotes chemotherapeutic resistance processes because it enhances multiple genetic alterations, which are usually detected in a more advanced tumor stage (60, 61). Then, under these environmental circumstances and after an apoptotic insult, naturally apoptosis-resistant malignant cells could be especially susceptible to necrotic-like forms of cell death.

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