Staurosporine-induced death of MCF-7 human breast cancer cells: a distinction between caspase-3-dependent steps of apoptosis and the critical lethal lesions

Liang-yan Xue, Song-mao Chiu, and Nancy L. Oleinick*

Department of Radiation Oncology, and the CWRU/Ireland Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

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Abstract

To test the role of caspase 3 in apoptosis and in overall cell lethality caused by the protein kinase inhibitor staurosporine, we compared the responses of MCF-7c3 cells that express a stably transfected CASP-3 gene to parental MCF-7:WS8 cells transfected with vector alone and lacking procaspase-3 (MCF-7v). Cells were exposed to increasing doses (0.15–1 μM) of staurosporine for periods up to 19 h. Apoptosis was efficiently induced in MCF-7c3 cells, as demonstrated by cytochrome c release, processing of procaspase-3, procaspase-8, and Bid, increase in caspase-3-like DEVDase activity, cleavage of the enzyme poly(ADP-ribose) polymerase, DNA fragmentation, changes in nuclear morphology, and TUNEL assay and flow cytometry. For all of these measures except cytochrome c release, little or no activity was detected in MCF-7v cells, confirming that caspase-3 is essential for efficient induction of apoptosis by staurosporine, but not for mitochondrial steps that occur earlier in the pathway. MCF-7c3 cells were more sensitive to staurosporine than MCF-7v cells when assayed for loss of viability by reduction of a tetrazolium dye. However, the two cell lines were equally sensitive to killing by staurosporine when evaluated by a clonogenic assay. A similar distinction between apoptosis and loss of clonogenicity was observed for the cancer chemotherapeutic agent VP-16. These results support our previous conclusions with photodynamic therapy: (a) assessing overall reproductive death of cancer cells requires a proliferation-based assay, such as clonogenicity; and (b) the critical staurosporine-induced lethal event is independent of those mediated by caspase-3.

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Introduction

Apoptosis is a programmed mechanism for cell death that is characterized by unique morphological and biochemical changes. This evolutionarily conserved cell death process is important for normal development and tissue homeostasis [1–3]. It also occurs during pathological conditions (e.g., Alzheimer’s and other neurodegenerative diseases), as well as in response to many cancer chemotherapeutic agents. All of the common pathways of apoptosis require the participation of caspases, proteases with specificity for short amino acid motifs terminating in a critical aspartate, at which cleavage occurs. Of the two most common types of pathways for apoptosis [4], one involves the activation of plasma membrane death receptors, resulting in the recruitment and activation of the initiator caspase, caspase-8. The other pathway type is dependent upon mitochondria for the release of cytochrome c and other factors into the cytosol. Once in the cytosol, cytochrome c binds to apoptosis activating factor 1 (APAF-1) and procaspase-9 to form apotosomes [5,6], complexes that promote autoproteolytic cleavage of procaspase-9 to generate active caspase-9 [7,8]. The initiator caspases in turn cleave and activate the executioner caspases, such as caspases-3, -6, and -7, that subsequently cleave key cellular substrates,
including those that lead to the prominent morphological changes of chromatin condensation and DNA fragmentation [9].

Caspase-3 is the major caspase involved in apoptosis induced by many agents [10]. There have been a number of studies addressing the importance of this caspase for apoptosis in response to agents such as TNF-α [11,12], Fas ligand [13], doxorubicin, etoposide [14,15], and RGD peptides [16], as well as staurosporine [11,12,17,18]. These studies have been facilitated by the finding that human breast cancer MCF-7 cells lack a functional CASP-3 gene and therefore do not express procaspase-3. The further availability of MCF-7 cells expressing a transfected human procaspase-3 (here termed MCF-7c3 cells) and the paired cells transfected with an empty vector (MCF-7v cells) has permitted study of the features of apoptosis that require functional caspase-3 during apoptosis. In an earlier study of photodynamic treatment (PDT) with the phthalocyanine photosensitizer Pc 4, we found that many common features of apoptosis, such as cleavage of poly(ADP-ribose) polymerase (PARP) and chromatin condensation, occurred efficiently in MCF-7c3 cells but only slowly and to a much lower extent in MCF-7v cells [19]. Furthermore, when cell viability was measured by a tetrazolium dye reduction assay, MCF-7c3 cells proved to be more sensitive to Pc 4-PDT than MCF-7v cells. However, the difference between the two cell lines disappeared when overall cell killing was evaluated with a clonogenic assay [19]. The findings indicate that caspase-3, while important for the final phases of apoptosis after Pc 4-PDT, is irrelevant in determining overall reproductive death of cells. Since Pc 4 accumulates in mitochondria [20] and produces specific photodamage to these organelles, including the photodestruction of Bcl-2 [21], it appears that the critical lethal events are distinct from the steps requiring caspase-3.

While that conclusion is valid for PDT, especially when mitochondria are primary targets, the relevance of the caspase-3-mediated apoptotic events for overall cell death following other types of agents is still not known. Previous studies have compared MCF-7 cells with and without overexpressed procaspase-3 with respect to the appearance of selected morphological and biochemical features of apoptotic cells [11–14,22], the cleavage of particular protein substrates [12,14,18,23–25], or the loss of cell viability, as measured by the trypsin blue or other short-term assays [10,11,22]. Such cell viability assays provide an incomplete assessment of cell killing for various reasons. First, they measure the loss of some particular cell function or inhibition of cell growth, but in fact do not measure cell killing directly. Second, they record events that have occurred up to the time of the assay but not those that may occur at later times. And third, they are limited to revealing up to about 90% loss of the measured function and are not reliable for greater levels of cell damage. For evaluation of the efficacy of a cancer therapy, information is required on the ability of an agent to reduce cell survival by several orders of magnitude. For this reason, clonogenic assays have become the gold standard for determining the fraction of cells surviving a particular treatment [26]. Unfortunately, none of the studies evaluating the role of caspase-3 compared the results on apoptosis to overall cell death, as determined by clonogenic assay. Therefore, we asked whether or not caspase-3 was needed for loss of clonogenicity when MCF-7 cells were treated with a specifically established apoptosis-inducing agent, staurosporine.

Staurosporine (STS) is a protein kinase inhibitor and a strong inducer of apoptosis [27,28]. The mechanism of apoptosis in response to STS is mediated by the translocation of Bax from the cytosol to mitochondria, mitochondrial dysfunction, and the release of cytochrome c [29–32]. A similar but slower appearance of apoptosis has been reported in STS-treated MCF-7 cells lacking caspase-3 [22]. Interestingly, in that study, cleavage of BID and procaspase-8, which are normally found in response to plasma membrane receptor activation, was observed in MCF-7 cells with functional caspase-3 but not in the cells lacking it. The finding implies that cross-talk between two apoptotic pathways can occur in the presence of caspase-3 and contribute to the accelerated apoptotic process. To better understand the role of caspase-3 in cell killing, we have compared the sensitivity of MCF-7 cells with and without functional caspase-3 toward staurosporine using a variety of measures, including clonogenic assays. A more limited analysis was conducted with the topoisomerase II inhibitor VP-16 [33] to extend the findings to a third apoptosis-inducing agent.

**Materials and methods**

**Cells and culture conditions**

The human breast cancer MCF-7 (WS8) cell line transfected with the pBabepuro retroviral vector encoding human procaspase-3 cDNA (here referred to as MCF-7c3 cells) or empty vector (MCF-7v cells) was derived by Dr. C.J. Froellig (Northwestern University, Evanston, IL) and provided to us by Drs. John Pink and David Boothman (Case Western Reserve University, Cleveland, OH). Both cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 2 μg/ml puromycin. Culture doubling times were approximately 24 h. The presence of procaspase-3 in MCF-7c3 but not in MCF-7v cells was confirmed by Western blot analysis [19].

**Clonogenic cell survival**

Cells were seeded into 25-cm² flasks and allowed to grow until the cell density reached 1–2 × 10⁶ cells per flask (50–60% confluency). A stock solution of STS (1 mM) was prepared in DMSO. Aliquots were added to the culture medium to give final concentrations of STS of 0.15–1 μM, and the cultures were incubated for various periods up to
19 h. For study of VP-16, 10 mM stock (in DMSO) was added to cells to give final concentrations of 0.5, 1, 2, 5, and 10 μM, and then the cells were incubated for 24 h. The cells were collected from the monolayer with trypsin, and aliquots were plated in triplicate into 6-cm petri dishes in amounts sufficient to yield 50–100 colonies per dish. After incubation for 13–14 days, the cells were stained with 0.1% crystal violet in 20% ethanol, and colonies containing at least 50 cells were counted.

Cell viability

Cells were seeded into 96-well microculture plates at 1 × 10^4 cells/well and allowed to attach overnight. The medium was removed and replaced with fresh medium with or without STS. The cells were incubated for various periods, after which cell viability was measured using the tetrazolium salt WST-1 (Boehringer Mannheim).

Fluorescence immunocytochemistry of cytochrome c

Cells grown on coverslips were stained as described [34]. Briefly, the coverslips were first incubated with mouse anti-cytochrome c antibody (1:300 dilution, clone 6H2.B4, Pharmingen, San Diego, CA) and then with second antibody, anti-mouse IgG conjugated to Texas red (Vector Laboratories). The coverslips were mounted on slides, examined with a Leitz fluorescence microscope, and photographed.

Determination of the yield of apoptotic cells by fluorescence staining of DNA

Cellular DNA was stained with 10 μg/ml Hoechst 33342 and examined under a fluorescence microscope. Apoptotic cells were determined by their nuclear morphology, i.e., condensed and fragmented chromatin. At least 200 cells were scored from each sample.

Pulsed-field gel electrophoresis (PFGE)

Total cellular DNA was isolated and analyzed for its size distribution as described previously [35].

Western blot analysis

Cells were lysed and sonicated, and cellular protein was denatured, as described [19]. Equivalent amounts of protein were loaded onto polyacrylamide gels, subjected to electrophoresis, transferred to a PVDF membrane, and incubated with a monoclonal anti-PARP antibody (Trevigen, Gaithersburg, MD), anti-caspase-3 monoclonal antibody (Transduction Laboratories, Lexington, KY), anti-caspase-8 antibody (Pharmingen), anti-Bid antibody (Santa Cruz, Santa Cruz, CA), or anti-actin antibody (Amersham, Arlington Heights, IL). The immune complexes were detected by enhanced chemiluminescence (ECL) system (Amersham).

Caspase activity

As previously described [19], caspase 3-like activity was measured using DEVD-7-amino-4-methylcoumarin (AMC) (BIOMOL Research Laboratory, Inc., Plymouth Meeting, PA) as a substrate.

Flow cytometry

Cell fixation and staining were performed based on the APO-DIRECT kit (Phoenix Flow Systems, Inc.). Analysis of fluorescein isothiocyanate (FITC)-tagged DNA and propidium iodide (PI) was carried out in the Flow Cytometry Facility of the Case Western Reserve University/Ireland Comprehensive Cancer Center [35].

Results

In order to study the importance of caspase-3 in the killing of cells by STS, we first characterized the dose response for apoptosis by several criteria, beginning with the TUNEL assay for detection of cells with fragmented DNA. Fig. 1 shows representative flow cytometry histograms for cells that were either untreated or treated with 0.7 or 1 μM STS for 19 h, as well as the estimated percentage of apoptotic cells for each condition. It is apparent that 0.7 μM STS did not induce significant apoptosis in MCF-7v cells, as measured by TUNEL positivity; however, 26% of the MCF-7v cells were observed to be apoptotic upon exposure to 1 μM STS. In contrast, 89 and 85% of MCF-7c3 cells were undergoing apoptosis after 0.7 and 1 μM STS treatment, respectively. The results indicate that caspase-3 promotes apoptosis induced by STS.

We next examined morphological changes in STS-treated MCF-7v and MCF-7c3 cells, as revealed by staining of the DNA with Hoechst 33342 and fluorescence microscopy. Representative images are shown in Fig. 2A, and the calculated percentages of nuclei with condensed chromatin in Fig. 2B. No apoptosis was observed in STS-treated MCF-7v cells, except for those treated with 1 μM STS for 17 h, which resulted in ~10% apoptosis (Fig. 2B). These results confirm those of Fig. 1. When MCF-7c3 cells were treated with STS for 1 h and immediately stained, no apoptotic cells were found (data not shown). However, postincubation of 1 h STS-treated MCF-7c3 cells in fresh medium for 16 h resulted in apoptosis (up to 30% for 1 μM STS; left panel in Fig. 2B). STS-induced apoptosis of MCF-7c3 cells increased in a time- and dose-dependent manner. The yield of apoptotic cells increased dramatically from 1 to 6 h and remained high at 17 h. The appearance of morphological apoptosis was completely prevented by the general caspase inhibitor zVAD-fmk (data not shown).
In order to further investigate the role of caspase-3 in STS-induced apoptosis, we used pulsed-field gel electrophoresis to study the early steps in DNA fragmentation. As originally described by Walker et al. [36], the first phase of DNA fragmentation involves the generation of large DNA fragments of 50 and/or 300 kbp. As shown in Fig. 3, a strong DNA band centering on ~50 kbp was observed in MCF-7c3 cells following 4 h of exposure to 1 μM STS, and a much weaker band was obtained from similarly treated MCF-7v cells. The results imply that the extent of apoptosis was much reduced in STS-treated MCF-7v cells as compared to MCF-7c3 cells due to the deletion of caspase-3.

A characteristic feature of apoptosis is proteolytic cleavage of specific cellular proteins, including PARP, by one or more caspases [37–39]. Accordingly, MCF-7v and MCF-7c3 cells were treated with various doses of STS for 17 h, and then total cellular protein was analyzed for PARP cleavage by Western blot (Fig. 4). For untreated cells of both lines, the majority of the PARP migrated at the position of the intact native enzyme (116 kDa). When MCF-7v cells were exposed to 0.15 or 0.3 μM STS, little or no PARP cleavage was observed, as indicated by faint bands at the position of the ~90-kDa cleavage product. At the higher STS doses, ~20% of PARP from MCF-7v cells was cleaved. Since these cells do not express caspase-3, the limited PARP cleavage may have resulted from caspase-7, which cleaves proteins at the same consensus sequence as caspase-3 [17,22]. For MCF-7c3 cells, the STS-dose dependence of PARP cleavage was more marked, such that treatment with 1 μM STS resulted in essentially 100% PARP cleavage. To check for concomitant activation of caspase-3, the same blot was reprobed with a monoclonal anti-procaspase-3 antibody. As shown in the middle panel of Fig. 4, no procaspase-3 was found in either untreated or STS-treated MCF-7v cells. However, for MCF-7c3 cells, there was a dose-dependent loss of procaspase-3, indicative of the proteolytic processing of the proenzyme to the active enzyme subunits, which are not detected by the antibody.

The activation of caspase-3 was also assessed by measuring enzyme activity with the synthetic fluorogenic substrate DEVD-AMC. MCF-7c3 cells were incubated with 0–1 μM STS for 1–17 h, and then caspase-3-like activity was assayed (Fig. 5). No DEVDase activity was found in cells treated with any dose for only 1 h. However, caspase-3 activity increased with STS dose and with time of exposure, reaching a maximum at 6 h and 1 μM. By 17 h, caspase-3 activity had decreased and was similar to that at 3.5 h. In contrast to the marked development of DEVDase activity in MCF-7c3 cells, there was little if any such activity in MCF-7v cells treated with the same concentrations of STS for 6 or 17 h (data not shown).

Fig. 6 shows the subcellular localization of cytochrome c
in control and STS-treated MCF-7v and MCF-7c3 cells using a fluorescence immunocytochemical method. In control cells, cytochrome c displays a perinuclear, punctate pattern, consistent with its location in mitochondria. As expected for cytochrome c release into cytosol, a diffuse fluorescence was observed in STS-treated cells of both lines, implying that the release of cytochrome c was not dependent on the presence of caspase-3.

The above data suggest that STS activates the mitochondrial pathway for apoptosis, for which caspase-3 is the primary executioner caspase. Another apoptotic signaling pathway is initiated by cell-membrane death receptors and

Fig. 2. Morphological changes in nuclei of STS-treated MCF-7v and MCF-7c3 cells. Cells were untreated or treated with 0–1 μM STS for 1, 6, or 17 h. At the end of the exposure period for 6 and 17 h or incubation in fresh medium for 16 h after a 1-h exposure to STS, cells were stained with Hoechst 33342 and observed under a fluorescence microscope. (A) Representative results from cells untreated or treated with 1 μM STS for 6 h. The percentage of cells containing nuclei with condensed chromatin was calculated (B). The diagram above each panel describes the experimental conditions. STS was added at Time 0 (△). Cells were stained with Hoechst 33342 either 6 or 17 h later (●). For the leftmost panel, the STS-containing medium was removed and replaced with fresh medium after 1 h (↑). Data in part B represent the mean and standard deviation of at least 200 cells from each culture and 3 experiments.
mediated by caspases-8 and/or -10. There is evidence for cross-talk between these pathways, such that caspase-8 can be activated by caspase-3, resulting in cleavage of the Bcl-2 homolog Bid, which can act on mitochondria to promote release of cytchrome c and activation of caspase-3 [22]. To test whether such an amplification mechanism may apply in the case of STS-treated MCF-7 cells, both cell lines were treated with 1 μM STS for 4 or 6 h, and then the processing of procaspase-8 and Bid was assessed by Western blot analysis. As shown in Fig. 7, there was no change in the levels of either procaspase-8 or Bid in STS-treated MCF-7v cells. In contrast, most of the procaspase-8 and Bid of MCF-7c3 cells were cleaved by 6 h after the addition of 1 μM STS. Our results are consistent with those of Tang et al. [22], and indicate caspase-3-dependent processing of procaspase-8 in response to STS.

We previously reported that caspase-3 is important for the efficient induction of apoptosis by PDT, but did not influence the critical lethal event. Since the mechanisms and targets of action of STS and PDT are different, it is possible that the distinction between lethal damage and caspase-3-dependent steps in apoptosis that we found for PDT may not apply to STS, whose primary action is presumed to involve inhibition of protein kinases. Therefore, we have assessed cell death by WST-1 tetrazolium dye reduction (Fig. 8) and by clonogenic assay (Fig. 9). As determined by WST-1
assay, MCF-7c3 cells were much more sensitive to STS than MCF-7v cells. The differential sensitivity was apparent throughout the range of STS doses that produced differential induction of apoptosis. By this criterion, the ability to carry out apoptosis seems to be critical to cell killing by STS. However, because the WST-1 assay does not measure

<table>
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<tr>
<th>MCF-7v</th>
<th>MCF-7c3</th>
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<td>1 μM STS</td>
<td>-</td>
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<tr>
<td>post (h)</td>
<td>-</td>
</tr>
<tr>
<td>Caspase-8</td>
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<td>Bid</td>
<td>-</td>
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<td>Actin</td>
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Fig. 6. Cytochrome c release in control and STS-treated MCF-7v and MCF-7c3 cells. Cells were untreated or treated with 1 μM STS for 6 h and then stained with anti-cytochrome c antibody for fluorescence immunochemistry.

Fig. 7. The effect of caspase-3 on cleavage of caspase-8 and Bid. MCF-7v and MCF-7c3 cells were treated with 1 μM STS for 4 or 6 h. Total cell lysates from control and treated cells were electrophoresed and transferred to PVDF membranes. The membranes were probed with anti-caspase-8 and anti-Bid antibodies and then reprobed with anti-actin as a loading control.
cell death directly and records only those events that have occurred by the time of the assay, it can misrepresent the overall death of cells. Therefore, we conducted a clonogenic assay, which measures the ability of each cell in the culture to maintain all the functions needed to divide and form a colony. Figure 9 shows the overall sensitivity of the two cell lines treated with various doses of STS for 1, 6, 12, or 19 h. When either cell line was exposed to 1 μM STS for 1 h, ~30% of the cells survived (i.e., ~70% cells were killed). With additional time of exposure up to 6 h, cell death was greatly increased (to 98.8 and 99.6% for MCF-7v and MCF-7c3, respectively). When the incubation time increased to 12 or 19 h, however, there was no further cell killing and the survival curves of cells treated with STS for 6, 12, or 19 h are nearly the same. D_{37} values for these survival curves are given in Table 1 and confirm that a 6-h treatment of either cell line with STS produces nearly the same degree of cell killing as a 19-h treatment. Comparing the data between the two cell lines, it is apparent that the overall sensitivity to STS differed only slightly. The results demonstrate that the presence of caspase-3 had little effect on the ability of STS to produce lethal damage, although it promoted the elimination of dead cells by apoptosis.

The above results confirm for a second apoptosis-inducing agent the distinction between the requirements for apoptosis and for reproductive cell death. To extend the observations to a third agent, we briefly evaluated the effects of the cancer chemotherapeutic drug, etoposide (VP-16). As shown in Table 2 and Fig. 10, this topoisomerase II inhibitor induces a higher level of apoptosis in MCF-7c3 cells than in MCF-7v cells, but the two cell lines are equally susceptible to VP-16, as determined by loss of clonogenicity.

### Discussion

In this study, the differential ability of MCF-7v and MCF-7c3 cells to undergo apoptosis in response to STS was demonstrated by TUNEL assay and flow cytometry (Fig. 1); nuclear morphology (Fig. 2); DNA fragmentation (Fig. 3); processing of procaspase-3 (Fig. 4), procaspase-8 and Bid (Fig. 6); increase in caspase-3-like activity (Fig. 5); and PARP cleavage (Fig. 4). This selection of assays provides ample evidence of the different responses of the two cell lines. Our data confirm the results reported by several other

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<th>Exposure</th>
<th>D_{37} (MCF-7v)</th>
<th>D_{37} (MCF-7c3)</th>
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<tr>
<td>6 h</td>
<td>0.21</td>
<td>0.25</td>
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<tr>
<td>12 h</td>
<td>0.20</td>
<td>0.19</td>
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<tr>
<td>19 h</td>
<td>0.18</td>
<td>0.19</td>
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* D_{37} values, the concentration of STS (μM) that reduces survival by 1/e, were calculated from the fitted curves of dose-response curves in Fig. 9 by Microsoft Excel, assuming an exponential increase in cell killing with STS dose.

Table 2

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<th>Cell line</th>
<th>% apoptotic cells ± standard deviation</th>
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<tr>
<td>MCF-7v</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>MCF-7c3</td>
<td>29.3 ± 7.7</td>
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*Note. The percentage of cells in morphological apoptosis was determined by fluorescence microscopy after staining with Hoechst 33342.
investigators, who showed that apoptosis in MCF-7 cells expressing procaspase-3 is more rapid and extensive than in the matched cells lacking this proenzyme [14,16,40]. However, despite the complete absence of procaspase-3 in MCF-7v cells, a small amount of PARP cleavage was noted after 19 h in 0.7 or 1.0 μM STS (Fig. 4). This was in contrast to the nearly complete loss of native PARP in MCF-7c3 cells. Although no DEVDase activity above the level found in untreated cells was noted in MCF-7v cells, a small amount of caspase-7 or -6 may have been responsible for cutting PARP [14,17,41]. The cleavage may also be the result of other as yet unknown caspases or noncaspase proteases that cut at approximately the same site during the cell death process. A caspase-3-independent pathway for apoptosis in STS-treated MCF-7 cells has been demonstrated by Janicke et al. [11,42].

The major question we have addressed is: Where is the point of no return in determining the fate of human breast cancer cells following damage by staurosporine? If the activity of caspase-3 is essential for killing MCF-7 cells, then cells lacking that enzyme should be highly resistant to cell killing when exposed to the drug; i.e., they should remain alive and proliferate indefinitely. On the other hand, if damage produced by STS at an earlier point in the response dooms the cell to die, then cell killing by STS should occur with or without caspase-3. In the latter case, a doomed cell may be systematically dismantled by caspase-3-dependent apoptosis when possible, or may employ alternative caspases [11,42], or may succumb to the STS-damage by cell lysis or ceasing growth or some other process. For studies of the effects of toxic agents on normal differentiated cells, such as neurons or cardiac muscle, reproductive potential is not an appropriate endpoint. However, as pointed out by Brown and Wouters [26], the clonogenic assay is the gold standard for evaluating this question for cancer therapies, because it integrates information on all cell death events of any type as well as records both early and late events. When this standard was applied to the case of STS or of VP-16 in human breast cancer cells, the results clearly demonstrated that killing of cells by either of these agents did not depend upon the ability of the cells to express caspase-3 activity, even though fewer cells lacking caspase-3 appeared to be dead when measured by the WST-1 assay. The irrelevance of functional caspase-3 in determining cell death toward other chemotherapeutic agents has also been reported [15]. We have previously proposed that the point of no return for PDT occurs at or near the step of cytochrome c release, since the release of cytochrome c occurs in both MCF-7 cell lines following PDT [19]. The release of cytochrome c not only would trigger apoptosis through caspase activation but also would result in mitochondrial dysfunction [43]. Either way could eventually lead to cell death. Cytochrome c is released after STS in both MCF-7 cell lines, indicative of mitochondrial damage. Thus, cells are committed to death once the mitochondrial pathway is activated, whether the downstream events are executed or not, and the release of cytochrome c is a good indicator of such activation.

Another interesting feature of the clonogenic assays is the information they provide concerning the kinetics of cell killing in response to STS. As shown in Fig. 9, a 1-h incubation of either cell line in STS (0.15–1 μM) produced only a partial response when compared to that of longer incubations (6–19 h). Similarly, a 1-h exposure of MCF-7c3 cells induced a suboptimal level of apoptosis (Fig. 2B). This may be explained if uptake of STS by the cells and inhibition of critical protein kinases require more than 1 h. However, a 6-h incubation was as effective as a 12- or 19-h exposure, indicating that the maximum lethal damage possible for each STS concentration was obtained by 6 h. Others have demonstrated that cell death, as measured by the appearance of apoptotic cells, by trypan blue uptake, or by standard TNF cytotoxicity, required continuous cell exposure to STS for periods up to 24 h [22, 42]. In those cases, shorter incubations resulted in less “cell death.” However, it now appears that the additional time in STS, beyond about 6 h, does not kill more cells but may promote amplification of apoptosis pathways, such as caspase-3-dependent activation of caspase-8 and cleavage of Bid, that do not occur at early times.

Caspase-8 and Bid were activated in our STS-treated MCF-7c3 cells, but not in MCF-7v cells. The marked cleavage of caspase-8 and Bid in STS-treated MCF-7c3 cells and the maximum caspase-3 activation occurring at 6 h posttreatment strongly suggest that these cleavages occur downstream of the cleavage of procaspase-3. Our results confirm the finding of Tang et al. [22] that there is cross-talk between the death receptor pathway and the cytochrome c/Apaf-1/caspase-9 pathway. Kotké et al. [44] also reported that the proteolytic cleavage of procaspase-8 was observed...
in MDA-MB-486 cells treated with paclitaxel, which might induce apoptosis through the cytochrome c pathway [45,46]. The activation of caspase-8 may shorten the execution time and accelerate cell death.

Over the past decade, there has been increasing interest in elucidating the role of apoptosis in determining the efficacy of cancer therapy. Most chemotherapeutic drugs and ionizing radiation have been shown to work, at least partially, by causing tumor cells to undergo apoptosis [26]. However, it is well known that some tumor cells are insensitive to the induction of apoptosis but can be killed through nonapoptotic mechanisms, e.g., necrosis, giant cell formation, or abnormal mitoses, whereas others are highly resistant to any form of cell death. The results of the present study, along with those of our previous one with PDT, suggest that simple evaluation of the ability of a drug or treatment to cause apoptosis is insufficient to evaluate its efficacy. Cell viability tests, such as tetrazolium dye reduction, are also too limiting in the information obtainable, because they can miss late apoptosis or cells dying by other mechanisms [26]. For the three agents we have evaluated (Pc 4-PDT, STS, and VP-16), it may be concluded that apoptosis is an efficient means of dismantling and eliminating dead cells but not the death process itself. Evaluation of other classes of agents should include clonogenic or other proliferation-based assays among the battery of tests employed.

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