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Calpains and Apoptosis

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Calpains are a family of cysteine proteases existing primarily in two forms designated by the Ca^{2+} concentration needed for activation *in vitro*, μ-calpain (calpain-I) and m-calpain (calpain-II). The physiological roles of calpains remain unclear. Many groups have proposed a role for calpains in apoptosis, but their patterns of activation are not well characterized. Calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis, as well as many other apoptotic pathways. Calpain activation in apoptosis is usually linked upstream or downstream to caspase activation, or in a parallel pathway alongside caspase activation. Calpains have been suggested to be involved in DNA fragmentation (via endonuclease activation), but also as effector proteases that cleave cellular proteins involved in DNA repair, membrane associated proteins and other homeostatic regulatory proteins. Recently, our laboratory demonstrated μ-calpain activation in NAD(P)H: quinone oxidoreducatse 1 (NQO1)-expressing cells after exposure to β-lapachone, a novel quinone and potential chemo- and radio-therapeutic agent. Increased cytosolic Ca^{2+} in NQO1-expressing cells after β-lapachone exposures were shown to lead to μ-calpain activation. In turn, μ-calpain activation was important for substrate proteolysis and DNA fragmentation associated with apoptosis. Upon activation, μ-calpain translocated to the nucleus where it could proteolytically cleave PARP and p53. We provided evidence that β-lapachone-induced, μ-calpain stimulated, apoptosis did not involve any of the known caspases; known apoptotic caspases were not activated after β-lapachone treatment of NQO1-expressing cells, nor did caspase inhibitors have any effect on β-lapachone-induced cell death. Elucidation of processes by which β-lapachone-stimulated μ-calpain activation and calpains ability to activate endonucleases and induce apoptosis independent of caspase activity will be needed to further develop/modulate β-lapachone for treatment of human cancers that over-express NQO1.

Cell Death

There is increasing evidence that classical apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths. Cytotoxic agents kill cells with similar features to both. These mechanisms are characterized by cellular morphology and an organism's biological response after cellular death has occurred. In necrotic cell death, dying cells essentially burst, spilling their internal contents into the intercellular space, resulting in an inflammatory response and further cellular damage (Bellamy et al., 1995). Apoptosis is a genetically programmed form of cell death with distinct signaling pathways. Programmed cell death (PCD, non-agent-induced apoptosis) is involved in development and normal turnover of cells (Meier et al., 2000). Similarly, apoptosis can be triggered by cellular insults resulting in a stimulation of PCD, but often times skipping a portion of PCD's genetic control (Kerr et al., 1972; Stepczynska et al., 2001). Once apoptosis is initiated, specific proteases (e.g., caspases and/or calpains) are activated resulting in biochemical and morphological changes in the cell. These changes include: DNA cleavage (TUNEL positive cell formation), chromatin condensation, nuclear fragmentation, cleavage of apoptotic substrates (e.g., PARP, lamin B), cytoplasmic membrane blebbing and formation of apoptotic bodies (small blebs of the membrane and cytoplasm) (Gerschenson and Rotello, 1992; Patel et al., 1996). Apoptotic bodies are subsequently phagocytosed by macrophages or neighboring cells without eliciting an inflammatory response, as opposed to cells that undergo necrotic cell death (Gerschenson and Rotello, 1992).

Apoptosis is a highly regulated and active process

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that requires the participation of endogenous cellular enzymes that systemically dismantle the cell. The most well characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases. However, other proteases are implicated in apoptosis as well (e.g., calpains). Apoptosis can be divided into two phases: initiation and execution. The induction of apoptosis via the caspases leads to a downstream signaling pathway that begins with the initiator caspases (e.g., caspases 8 and 9), depending upon the triggering agent. The initiation phases of apoptosis are not entirely understood, but are clearly agent-specific. These initiator caspases, in turn, activate downstream effector caspases (e.g., caspases 3, 6, and 7) (Kuida et al., 1998; Stennicke et al., 1998). Caspases exist as zymogens, inactive proenzymes that are proteolytically cleaved to form active tetramers (reviewed in (Nicholson, 1999). The effector caspases systemically dismantle the cell by cleaving proteins that are important in DNA repair (e.g., PARP, ATM and DNA-PKcs) (Kaufmann, 1989; Lazebnik et al., 1994; Casiano et al., 1996), cellular integrity (e.g., lamins, gelsolin and fodrin)(Vanags et al., 1996; Kothakota et al., 1997; Janicke et al., 1998) and cellular homeostasis (e.g., pRb)(Janicke et al., 1996), as well as by activating other apoptotic proteases (e.g., calpains) (Wang et al., 1998; Wood and Newcomb, 1999; Kato et al., 2000), that ultimately results in apoptotic cell death.

Calpains

Other proteases have been implicated in apoptosis, but their exact mechanisms of activation and intracellular targets are not well known. One of these proteases is the Ca^{2+}-activated neutral protease, calpain (EC 3.4.22.2). Calpains are a family of Ca^{2+}-dependent cysteine proteases that catalyze proteolysis of a limited number of proteins involved in cytoskeletal remodeling and signal transduction. Calpains are also implicated in a number of physiological and pathophysiological processes. These processes include cell cycle regulation, apoptosis, necrosis, muscular dystrophies, cataractogenesis, Alzheimer's and Parkinson's diseases (Goll et al., 1992; Nixon et al., 1994; Johnson and Guttmann, 1997; Sorimachi et al., 1997; Carafoli & Molinari, 1998; Richard et al., 1999).

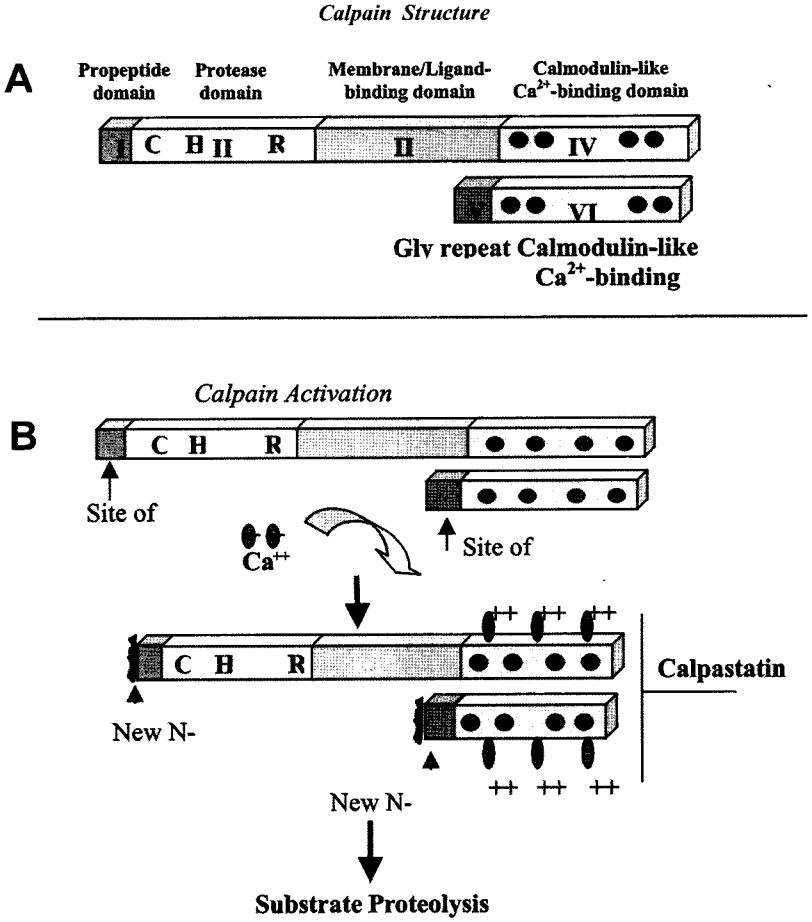

Fig. 1. A schematic diagram of calpain activation. See text for discussion.

Calpains exist primarily in two forms, designated by the Ca^{2+} concentration needed for activation *in vitro*: μ-calpain (calpain-I) and m-calpain (calpain-II). Each isoform is a heterodimer consisting of a large catalytic and a small regulatory subunit, both of which are sensitive to changes in Ca^{2+} homeostasis (Fig. 1). μ- and m-Calpains are ubiquitously expressed and predominantly located in the cytoplasm (Yoshimura et al., 1984; Banik et al., 1991), but can translocate to cellular membranes where they appear to become activated (Kawasaki and Kawashima, 1996), or to the nucleus during specific apoptotic insults (Tagliarino, et al. unpublished data).

Calpastatin, an inhibitor of μ- and m- calpains

Calpastatin is a specific endogenous inhibitor of m- and μ-calpains that binds calpains in the presence of Ca^{2+} (Fig. 2) (Cottin et al., 1981; Crawford et al., 1993). Calpastatin inhibits μ- or m-calpain in a substrate-competitive manner, and also inhibits calpains association with membranes (Maki et al., 1988; Kawasaki et al., 1989). Binding of calpains to calpastatin is reversible and does not result in any lingering loss of calpain activity (Kapprell and Goll, 1989).

Calpains have two distinct sites for binding to calpastatin, one at the active site and another at the EF-hand domain (Kawasaki and Kawashima, 1996). It is believed that calpain interacts with substrates through these same two sites.

Calpains in apoptosis

The physiological roles of calpains remain unclear (Carafoli and Molinari, 1998; Ono et al., 1998). The presence and conservation of m- and μ- calpains in almost all mammalian cells suggest that these enzymes are essential, however, the absence of specific calpain inhibitors has thus far prevented unambiguous proof of a particular physiological role. Many of the calpain inhibitors used in earlier work have been shown to also inhibit the proteosome, cathepsins, other cysteine proteases, or inhibit entirely different enzymes, for example, a protein tyrosine phosphatase (Barrett et al., 1982; Schoenwaelder and Burridge, 1999; Tenev et al., 2001). The calpain genes have no major regulatory features in their promoter regions and are usually considered to be housekeeping enzymes. The relative levels of m- and μ- calpains and of their inhibitor, calpastatin, vary from tissue to tissue, again suggesting some degree of regulation and importance in the cell (Thompson and Goll, 2000).

Calpains were first implicated in N-methyl-D-aspartate (NMDA)- and ischemia-induced apoptosis in 1993 (Roberts-Lewis et al., 1993). Since then, many other groups have proposed a role for calpains in apoptosis, but their patterns of activation are not well characterized; calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis,

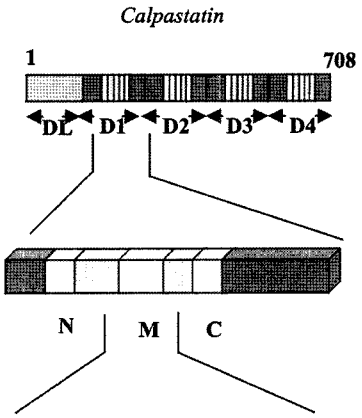

Fig. 2. A schematic diagram of calpastatin and its inhibitory domains. See text for discussion.

as well as many other apoptotic pathways (Squier and Cohen, 1997; Patel and Lane, 1999; Squier et al., 1999). Calpain activation in apoptosis is usually linked upstream or downstream to caspase activation, or in a parallel pathway alongside caspase activation (Wang et al., 1998; Wood et al., 1998; Ruiz-Vela et al., 1999; Wood and Newcomb, 1999). Caspases have been reported to be upstream of calpain activation; caspases (3 or 7) cleaved the endogenous inhibitor of calpain, calpastatin, thus allowing for increased calpain activation after apoptotic induction by anti-Fas or staurosporine exposures (Wang et al., 1998; Kato et al., 2000). Others have reported calpain activation upstream of caspase activation, demonstrating that calpains specifically trigger activation and processing of caspase 7 or 12 in various model systems (Ruiz-Vela et al., 1999; Nakagawa and Yuan, 2000). In addition, calpain may be responsible for cleaving the loop region in Bcl-xL and, therefore, turning the anti-apoptotic Bcl-xL protein into a pro-apoptotic molecule that further promotes caspase activation (Nakagawa and Yuan, 2000). Calpains can also inactivate caspases (3, 7, 8, or 9) by cleavage of the pro-caspase form to proteolytically inactive fragments (McGinnis et al., 1999; Chua et al., 2000). In addition, calpain activation in apoptosis can occur concurrently with, or in the absence of, caspase activation (Nath et al., 1996b; McGinnis et al., 1998; Okuno et al., 1998; Drenou et al., 1999; Wolf et al., 1999; Eby et al., 2000; Lankiewicz et al., 2000).

Under certain circumstances, calpain inhibitors blocked all aspects of apoptosis, including DNA fragmentation (Squier et al., 1994; Vanags et al., 1996; Squier and Cohen, 1997). These data imply that calpain activation led to endonuclease activation either directly, or indirectly. However, it is unclear whether this endonuclease activation occurred through activation of a caspase, another protease, or directly by calpains. Others have

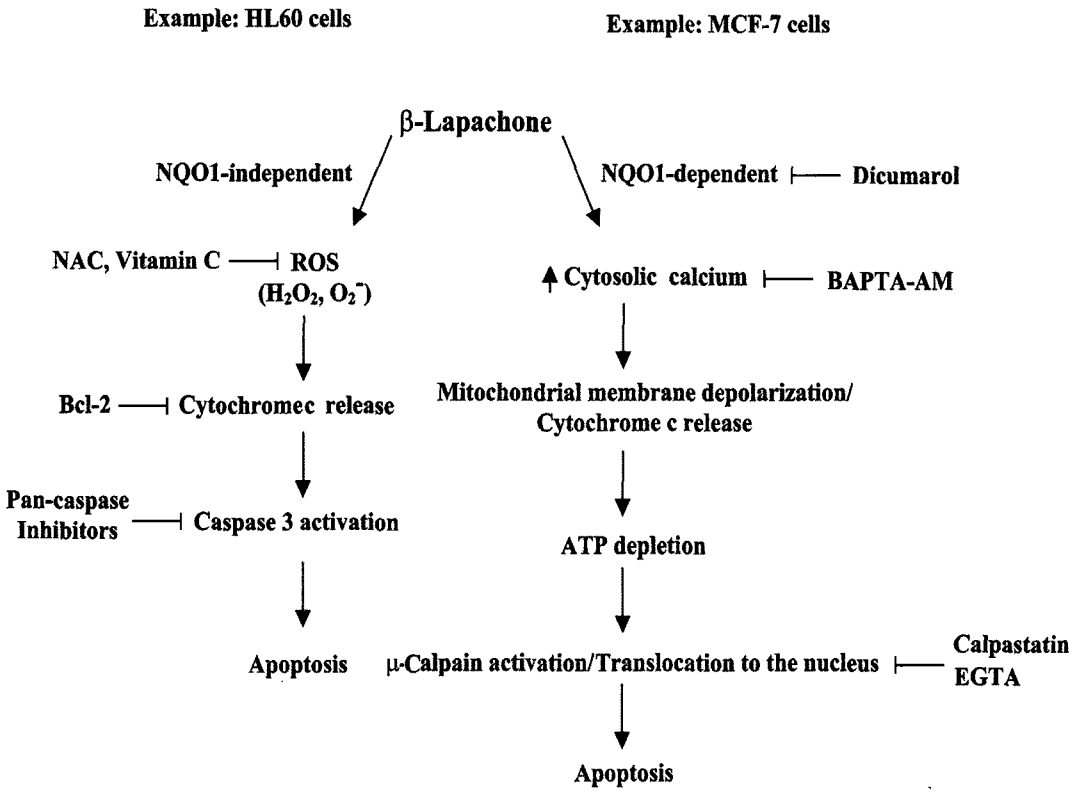

Fig. 3. A model of β-lapachone-induced apoptosis in NQO1-deficient and NQO1-containing cells. Cumulative data indicate that two separate pathways of apoptosis can be induced by β-lapachone. The expression of NQO1 is a key determinant of both the cell death pathway and dose of β-lap required to induce the apoptotic pathway. When cells express NQO1, the cell death pathway stimulated is Ca^{2+}-dependent, blocked by dicumarol and appears to involve the activation of calpain (see text). In the absence of NQO1, cell death appears to occur via a reactive oxygenmediated pathway that is caspase-mediated, and therefore prevented short-term by pan-caspase inhibitors and long tern by over-expression of bcl-2 (see txt). In figure ⊣ or ⊢ represent inhibitory effects, arrows represent stimulatory effects.

demonstrated that calpain inhibitors, but not caspase inhibitors, blocked DNA fragmentation and apoptosis, further implicating a potential role for calpain in endonuclease activation (Squier and Cohen, 1997; Villa et al., 1998). The formation of higher molecular weight DNA fragmentation was inhibited by calpain inhibitors in TNF-induced apoptosis in U937 cells, but was not affected by caspase inhibitors (Vanags et al., 1996). Since calpain inhibitors had no effect on caspase-fluorogenic substrate cleavage, it was suggested that caspases were upstream of calpains and calpain was an effector leading to extra-nuclear and nuclear endpoints (Vanags et al., 1996).

Calpain activation was demonstrated in thymocytes after apoptotic induction by irradiation or glucocorticoid exposures (Squier et al., 1994). Calpain activation preceded apoptotic morphology (DNA fragmentation and nuclear condensation) in dexamethasone-treated thymocytes (Squier and Cohen, 1997). Calpain inhibitors blocked all aspects of dexamethasone-induced cell death, including loss of membrane integrity, decreased cell volume, nuclear collapse and DNA fragmentation (Squier and Cohen, 1997). These data strongly suggest a role for calpains in apoptosis, or their role upstream of caspase activation under certain cellular stress conditions.

Calpains are not only suggested to be involved in DNA fragmentation (via endonuclease activation), but are also effector proteases that cleave cellular proteins involved in DNA repair (e.g., PARP(Buki et al., 1997; McGinnis et al., 1999), membrane associated proteins (e.g., α-spectrin and actin, (Nath et al., 1996b; Villa et al., 1998) and other homeostatic regulatory proteins (e.g., cyclin D1, c-FOS, C-JUN, p53, (Watt and Molloy, 1993; Langenfeld et al., 1997; Pariat et al., 1997; Squier and Cohen, 1997; Carafoli and Molinari, 1998). Calpain substrate cleavage does not involve a specific primary cleavage site, but rather, is dependent upon the secondary structure of the substrate, making calpains a class of proteases different from the aspartate-specific caspases (Croall et al., 1986; Sakai et al., 1987; Wang et al., 1989; Nath et al., 1996b).

Calpains have been implicated in a number of apoptotic pathways. Calpain-like activity in the mitochondria was found to cleave the pro-apoptotic molecule bax. Calpain inhibitors, in turn, inhibited bax cleavage, but did not affect PARP cleavage in HL-60 cells treated with 9-aminocamptothecin, a topoiso-

merase I inhibitor (Wood et al., 1998). Calpains were also involved in calphostin C-induced apoptosis; apoptosis induced by calphostin C in U937 human promonocytic leukemia cells was inhibited by a calpain/proteosome inhibitor, but not by a proteosome-specific inhibitor, further suggesting a role for calpain in apoptosis induced by this agent (Spinedi et al., 1998). Calphostin C is a potent and selective protein kinase C inhibitor in U937 cells (Spinedi et al., 1998).

Both necrotic and neuronal apoptotic cell death were observed in various neurological and neurodegenerative disorders (Wang, 2000). Calpain was activated under various necrotic and apoptotic conditions, while caspase 3 was only activated in neuronal apoptosis (reviewed in Wang, 2000). Despite the difference in cleavagesite specificity, an increasing number of cellular proteins were found to be dually susceptible to both calpains and caspase cysteine proteases. These included alpha- and beta- fodrin, calmodulin-dependent protein kinases, PARP and tau (Nath et al., 1996b; Canu et al., 1998; McGinnis et al., 1998; McGinnis et al., 1999). Neurotoxic challenges such as hypoxia-hypoglycemia, excitotoxin treatment or metabolic inhibition of cultured neurons resulted in activation of both proteases (Nath et al., 1996a; McGinnis et al., 1998; Rami et al., 2000). Calpain inhibitors protected against necrotic neuronal death and to a lesser extent, apoptotic death (Nath et al., 1996a), while caspase inhibitors strongly suppressed apoptotic neuronal death (Nath et al., 1996a). In cell necrosis (e.g., maitotoxin-treated neuroblastoma SH-SY5Y cells) alpha-spectrin breakdown products of 150 kDa and 145 kDa were produced by cellular calpains (Nath et al., 1996b). In contrast, in neuronal cells undergoing apoptosis (cerebellar granule neurons subjected to low potassium and SH-SY5Y cells treated with STS), an additional breakdown product of 120 kDa was observed that was caspase-mediated (Nath et al., 1996b). Inhibition of either caspases or calpains protected both granule neurons and SH-SY5Y cells against apoptosis (Nath et al., 1996b). These data further suggest an interaction between caspases and calpains in apoptosis and neurodegenerative conditions and furthermore, specific intracellular proteins are targeted during cell death processes by both cysteine proteases.

Calpains in β-lapachone-mediated apoptosis

β-Lapachone is a naturally occurring quinone present in the bark of the South American Lapacho tree. The drug has anti-tumor activity against a variety of human cancers, including colon, prostate, promyelocytic leukemia and breast (Li et al., 1995; Planchon et al., 1995; Wuerzberger et al., 1998). β-Lapachone was shown to be an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with low level host toxicity (Li et al., 1999). We recently demonstrated that β-lapachone killed human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H: quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (Pink et al., 2000a; Planchon et al., 2001). β-Lapachone cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (Pink et al., 2000a; Planchon et al., 2001). NQO1, a cytosolic enzyme elevated in breast cancers (Marin et al., 1997), catalyzes a two-electron reduction of quinones (e.g., β-lapachone, menadione), utilizing either NADH or NADPH as electron donors. We recently demonstrated that reduction of β-lapachone by NQO1 leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (Pink et al., 2000a). We also demonstrated that increases in intracellular Ca^{2+} levels were critical for the apoptotic pathway induced by β-lapachone (Tagliarino et al., 2001). Increased cytosolic Ca^{2+}, due to ER Ca^{2+} pool depletion, led to loss of mitochondrial membrane potential, ATP depletion, specific and unique substrate proteolysis, DNA fragmentation and cell death by apoptosis (Tagliarino et al., 2001). Increased cytosolic Ca^{2+} also led to μ-calpain activation and cytoplasm to nucleus translocation that was essential for substrate proteolysis and DNA fragmentation (Tagliarino, et al. unpublished data).

In β-lapachone-treated, NQO1-expressing cells, proteolytic cleavage of PARP correlated well with cell death, as well as classic Lamin B cleavage. However, PARP cleavage did not generate the classic caspase 3/7-mediated 89 kDa fragment. Instead, an ~60 kDa PARP polypeptide fragment was observed. This atypical fragment was most apparent in MCF-7 cells that were more sensitive to β-lapachone, due to elevated NQO1 levels, as well as in prostate cancer cells that over-expressed NQO1 (Pink et al., 2000a; Pink et al., 2000b; Planchon et al., 2001). Atypical PARP cleavage was inhibited by the global cysteine protease inhibitors, iodoacetamide and N-ethylmaleimide (Pink et al., 2000b). Iodoacetamide and N-ethylmaleimide react directly with active site cysteines and thereby inhibit all cysteine proteases, as well as other enzymes that contain accessible SH groups. In contrast, atypical PARP cleavage was insensitive to inhibitors of Granzyme B, caspases, cathepsins B and L, trypsin, and chymotrypsin-like proteases (Pink et al., 2000b). However, extracellular Ca^{2+} chelators, EGTA and EDTA, blocked these proteolytic events in a dose-dependent manner (Pink et al., 2000b). Furthermore, in NQO1-expressing prostate cancer cells, zVAD did not block PARP, p53 or lamin B cleavages after a 4 h pulse of β-lapachone (Planchon et al., 2001). Specifically, caspase 3 was not involved in β-lapachone-mediated cell death, since caspase 3-null and reconstituted MCF-7 cells showed no difference in cell death or proteolytic cleavages, implicating a caspase-

independent apoptotic cell death pathway (Pink et al., 2000b).

Recently, our laboratory demonstrated that β-lapachone-induced activation of μ-calpain in NQO1-expressing human breast cancer cells (Tagliarino, et al. unpublished data). In turn, β-lapachone-induced μ-Calpain activation mediated cleavage of p53 in a manner similar to that previously reported (Kubbutat and Vousden, 1997; Pariat et al., 1997), as well as a novel cleavage of PARP to an ~60 kDa polypeptide fragment. PARP is a caspase 3 substrate, and a widely used indicator of apoptosis when cleaved to a characteristic 89 kDa fragment from its 113 kDa full-length protein. PARP was previously reported to be cleaved by calpains to a 40 kDa fragment during maitotoxin-induced necrosis (McGinnis et al., 1999). PARP was also cleaved by calpains purified from calf thymus to ~42 kDa, ~55 kDa (doublet) and ~67 kDa (triplet) fragments (Buki et al., 1997). We demonstrated that purified μ-calpain cleaved ^{35}S-methionine *in vitro* transcribed and translated PARP to the same ~60 kDa fragment found in extracts from cells treated with β-lapachone, menadione, or ionomycin (Tagliarino, et al. unpublished data). This ~60 kDa PARP cleavage fragment was identical to the fragment observed via Western blot analyses of β-lapachone-treated, NQO1-expressing breast cancer cells (Tagliarino, et al. unpublished data). Calpain activity observed in drug-exposed cell extracts in *in vitro* assays was inhibited by a calpastatin peptide in a dose-dependent manner (Tagliarino, et al. unpublished data). Furthermore, the apoptotic responses in NQO1-expressing cells to β-lapachone were significantly delayed, and survival enhanced, via the exogenous over-expression of calpastatin, a natural calpain inhibitor (Tagliarino, et al. unpublished data).

Upon activation, m-calpain translocated to the nucleus where it could proteolytically cleave PARP and p53 (Tagliarino, et al. unpublished data). We provided evidence that suggests that β-lapachone-induced, μ-calpain stimulated, apoptosis did not involve any of the known caspases; Western blot analyses of β-lapachone-exposed breast cancer cells for apoptotic caspases (3, 6, 7, 8, 9, 10 and 12) demonstrated no change in full-length protein nor did they exhibit formation of the activated proenzyme fragment, 6-24 h after β-lapachone exposures (Tagliarino, et al. unpublished data).

A number of studies regarding the effects of β-lap on cells have also been performed in cells that are deficient in NQO1 (e.g., HL60 cells). In contrast to NQO1-mediated processes elicited by β-lap (discussed above), β-Lap induced caspase-mediated apoptotic responses at higher doses in cells deficient in NQO1. In NQO1-deficient cells, we propose that β-lap undergoes two-one electron reductions to the hydroquinone through a semi-quinone intermediate. This may lead to ROS that would be involved in an NQO1-independent cell death pathway. Chau et al. found a dramatic elevation of H_2O_2 in human leukemia HL-60 cells following 1 μM β-lap treatment and this increase was effectively inhibited with antioxidants *N*-acetyl-L-cysteine (NAC), ascorbic acid, and a-tocopherol (Chau et al., 1998; Shiah et al., 1999). NAC prevented apoptotic characteristics of DNA fragmentation and apoptotic morphology. Over-expression of ectopic bcl-2 in HL-60 cells also attenuated β-lap-induced H_2O_2 and conferred resistance to β-lap-induced cell death (Chau et al., 1998). Studies by Planchon et al. also demonstrated that bcl-2 over-expression in HL-60 cells prevented all aspects of β-lap-mediated cytotoxicity including an enhancement of survival of β-lap-treated cells (Planchon et al., 1999). β-Lap-induced apoptosis in HL-60 cells was accompanied by activation of caspase 3 and classic PARP cleavage that was blocked by caspase-specific inhibitors (Planchon et al., 1999). Over-expression of bcl-2 prevented β-lap-mediated caspase 3 activation and PARP cleavage while increasing viability of bcl-2 expressing cells, compared to vector alone (Planchon et al., 1999).

Therefore, there are two pathways of β-lapachone-mediated apoptosis dependent upon the NQO1 status of the cell type assayed. In β-lapachone-mediated, NQO1-dependent cell death μ-calpain is the primary protease and in NQO1-deficient cells, caspases are the predominant pathway mediating apoptosis. However, we have observed that NQO1-mediated cell death via μ-calpain activation was the dominant cell death pathway following β-lap exposures using genetically matched human breast (Pink et al., 2000a; Tagliarino et al., 2001) or prostate (Planchon et al., 2001) cancer cells.

In conclusion, calpains have been implicated in a number of apoptotic and cell death pathways, however, their pattern of activation as well as their association with caspases in these pathways, remains controversial. Further work on the ability of calpains to activate endonucleases, induce apoptosis independent of caspase activity, and their distinct role in cell death needs further elucidation. More importantly, the caspase- and p53- independent nature of calpain-mediated cell death induced by β-lapachone or various β-lapachone analogs must be further explored to (a) elucidate the unique signal transduction pathway occurring during this unique form of apoptotic cell death; and (b) exploit this cell death pathway for the treatment of human malignances, especially cancers that have lost caspase or p53 functions via accumulated mutations.

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