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Reactive Oxygen Species and Redox-induced Programmed Cell Death Due to MK 886: Cells ("Soil") "Trump" Agent ("Seed")

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Abstract. *Micromolar concentrations of the five-lipoxygenase inhibitor, MK 886 induce a "type 1" (apoptotic, extrinsic, death domain, receptor-dependent, caspase-positive) form of programmed cell death in Bcl-2-positive U937 human monoblastoid and HL-60 myeloid leukemia cells. A "type 2" (intrinsic, mitochondria-dependent, autophagic, in some examples caspase-negative (Panc-1)) form is induced in Panc-1 pancreatic and PC3 prostate cell lines. The latter two lines from epithelial-derived solid human cancers are Bcl-2-negative. Micromolar MK 886 induces an acute rise in Ca^{2+} in washed, Ca^{2+}-poor U937 and HL60 cells in Ca^{2+} and Mg^{2+}-free Hank's buffer. In U937 cells, much of the increase, or more properly redistribution, is nuclear in location (HL-60 not tested). No MK-886-induced acute Ca^{2+} increase developed in Panc-1 or PC3 cells. Bcl-2-positive HeLa cervical cancer cells exhibited an acute MK 886-induced increase in Ca^{2+}. In the U937, PC3 and Panc-1 cells examined, MK-886 rapidly increased oxidative stress and decreased mitochondrial membrane potential, indicating that neither event is directly determinative for the altered distribution of Ca^{2+} or the form of PCD observed. Inhibition of increased U937 Ca^{2+} by the anti-oxidant, N-acetyl-L-cysteine, the effects of inhibitors of mitochondrial function including antimycin A, atractyloside, cyclosporin A, the L/N channel blocker loperamide, the intracellular chelator BAPTA and 2 agents, HA-14 and 3-methyl-antimycin A3 that impair Bcl-2 function further define these events. These differences in the Ca^{2+} response and possibly also the form of PCD that results may depend upon the presence of Bcl-2 or a related protein participating in a juxta-nuclear / nuclear Ca^{2+} ion channel. The role of mitochondria, the mechanism by which increased oxidative stress initiates the rapid release of Ca^{2+} from intracellular, possibly juxta-nuclear / nuclear sites or its redistribution to U937 Ca^{2+} nuclei, and whether this "signal" or possibly even ROS themselves mandate the type of PCD observed, presumably by differential modulation of transcription, remain to be determined. Lastly, these results demonstrate that, as might be expected, "soil" (cell type) trumps "seed" (inciting agent)".*

Abbreviations: BAPTA-AM, 1,2-bis(2-amino-5-fluorophenoxy (ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl) ester; CRE, cyclic AMP response element binding protein; CREB, cyclic AMP response element binding protein; DCDHF, 2',7',-dichloro,dihydro-fluorescein; 5-LPox, 5-lipoxygenase enzyme; IP3, 1,4,5,tris-inositol phosphate and related congeners; IP3R, IP3 receptors; MAPK, MAP kinase; MM, mitochondrial membrane; MMP, mitochondrial membrane potential; MMPT, mitochondrial membrane permeability transition; MPT, mitochondrial permeability transition; NF-AT, nuclear factor for activating transcription; PCD, $PCD_{1 \text{ or } 2}$, programmed cell death; RNS, reactive nitrogen species; ROS, reactive oxygen species; SRE, serum response element (Fura-2, Rhod-2, DCDHF; fluorescent probes).

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Key Words: MK 886, programmed cell death, Bcl-2, U937 nuclear Ca^{2+}.

Introduction

In the hematopoietic and non-hematopoietic cell lines examined, disparate forms of PCD were induced by MK 886. MK 886 is a lipophilic inhibitor of 5-lipoxygenase at nanoM concentrations, which at microM concentrations induces a type 1, "apoptotic" (extrinsic, death-domain, receptor / ligand-dependent, caspase-active) form of programmed cell death (PCD)) in Bcl-2-positive U937 and HL60 hematopoietic cells (1-3). The agent induces an atypical, non-apoptotic form of PCD, provisionally denoted as a "type 2" (intrinsic, autophagic, mitochondria-dependent, in some instances caspase-negative) form in human Bcl-2-negative PC3 prostate (4) and Panc-1 pancreatic (5) cancer cell lines (Table I). It is of interest that non-hematopoietic Panc-1 and PC3 lines can also express a type 1 apoptotic programmed cell death induced by agents such as

Table I. *Some features characterizing Panc-1 cells cultured with MK 886, actinomycin D or their combination (5).*

# Attribute	+MK	+ Act D	MK + Act D
Cytoplasmic			
1. Microfibrillar network[1]	Minimized	Variable bundles	Variable bundles
2. Cytoplasmic organelles	Normal distribuition	Regionalized	Regionalized
3. Lamellar bodies	Increased #s	Increased AC[2]	Increased #s
4. Mitochondria	Var. #s, distorted	High #s, distorted	Few, high #s distorted
5. Cisternae network	Extensive chanellin	Extensive swelling	Ext. Swelling
6. RER cisternal network	Swollen, extensive	Swollen	Stacked arrays
7. Polysomes	Reduced	Normal	Variable
8. Ribosomes (free)	Increased #s	Few/normal #s	Few/normal #s
Nuclear			
1. Chromatin condensates	Normal	Increased	Increased
2. Fragmented nuclei	Normal	Increased	Increased
3. Nuclear bodies	Increased #s	Few/normal #s	Few /normal #s

[1]Cytoskeletal elements + fibrils; [2]AC, type 1 apoptotic cells.

Table II. *Some responses of cells to MK 886 (2,4,5,8,9).*

	Bcl-2	Cell Ca^{2+}	Reduced MMP	Increased ROS	Caspase activity
Hematopoietic cells					
U937*	+	+	+	+	+
HL 60	+	+	+	+	ND
Non-hematopoietic cells					
PC-3	-	-	+	+	ND
Panc-1	-	-	+	+	-
HeLa	+	+	ND	ND	ND

ND, not done; +, present, increased fura-2 fluorescence or caspase activity; -, absent, no change in fluorescence or reduced caspase activity; MMP = mitochondrial membrane potential.
* , fluorescence primarily nuclear in location. HL60 not yet studied.

actinomycin D (6, Panc-1) and SC-41661A , a redox-dependent 5-LPox inhibitor (4; PC3). Table I lists some major ultrastructural differences exhibited by Panc-1 cells exposed to actinomycin D or MK 886 that distinguished these outcomes. What differences in mechanism account for the disparate PCD-responses to MK 886 in these solid and hematopoietic cell lines?

MK-886 rapidly increased Ca^{2+} (estimated by fura-2 fluorescence) in U937 but not in PC3 and Panc-1 cells. To identify MK886-induced intracellular Ca^{2+} fluxes, cells were washed in Ca^{2+} and Mg^{2+}-free Hank's buffer, and loaded and incubated with Fura-2-AM in the same buffer (2,7,8). Fluorescence increased only in U937 cells and subsequently much of it was found by confocal microscopy to be localized primarily in nuclei (Figure 1, Table II), further verified by the "Z-stacking" technique (8). The increased Ca^{2+}, which is due to an intracellular re-distribution, was not synchronous and most nuclei retained their fluorescence during the initial 5 min of observation. Over time, further redistribution and loss of fluorescence occurred, ascribed in part to the non-physiologic conditions and agonal events. Within 10-20 sec of MK 886, fluorescence increased, was inhibited by the antioxidant, N-acetyl-L-cysteine, identifying a primary role for oxidation / reduction in the mechanism of release, and by pretreatment with the intracellular Ca^{2+} chelator, BAPTA. The apparent release and redistribution of cellular Ca^{2+} was not associated with an evident reduction in cytoplasmic fluorescence. Loading cells with the more lipophilic, mitochondrial membrane permeability/charge Ca^{2+} probe, Rhod-2-labelled mitochondria and, interestingly, nucleoli but not the nucleoplasm. After MK 886, fluorescence at both locations remained unchanged.

MK 886 raises Ca^{2+} in Bcl-2-positive HeLa cells. In Bcl-2-positive HeLa (human cervical carcinoma) cells, MK 886 induced a Ca^{2+} signal (Figure 2, ref. 9.). Increased Ca^{2+} due to 10 mM ionomycin was augmented further by MK 886, while the L/N Ca^{2+} channel blocking agent loperamide at 10 mM strongly inhibited the response to MK 886.

Evidence that MK 886 induces increased reactive oxygen species. MK-886 rapidly caused a general increase in DCDH-fluorescein (DCDHF) fluorescence, representing formation of reactive oxygen species (ROS). This result occurred in U937, PC-3 and Panc-1 cells examined with flow cytometry (2). In U937 cells studied with confocal microscopy, the changes in ROS among the population of cells varied

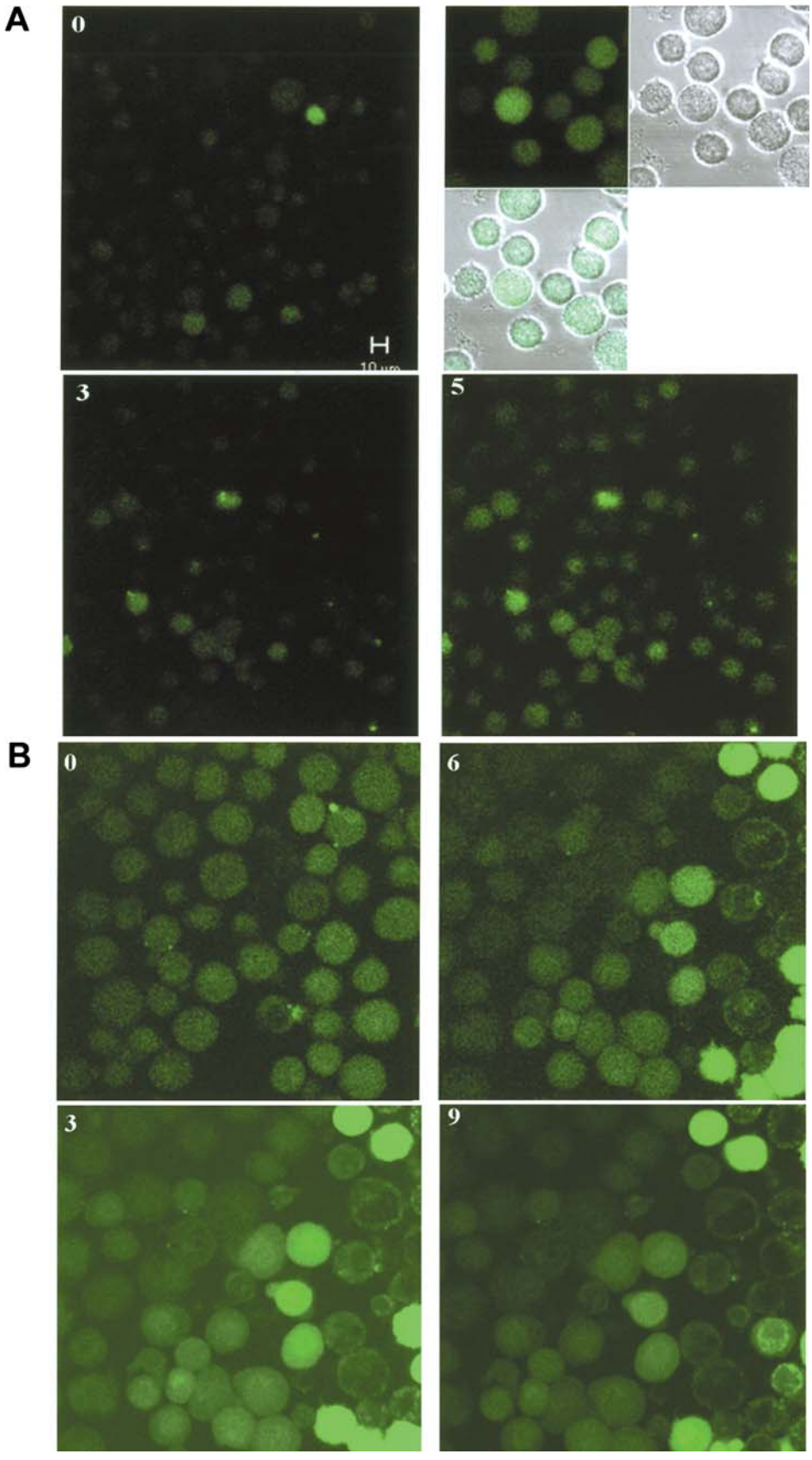

Figure 1. *A. (left panels, 0,3 and 5 min). Imaging of U937 cells in Ca^{2+} - free medium exposed to 40 µM MK 886 and examined with a Zeiss confocal microscope (8). Within 30 sec a uniform "nuclear" fura-2-dependent fluorescence without evident peri-nuclear or cytosolic enhancement appeared overlying a number of affected nuclei. Increases were not synchronous and most nuclei retained their fluorescence during the period of observation. "0"-, 3- and 5- min images are presented (x 250). Insert, phase contrast overlays (x 400). B. (right panels, 0,3,6,9 min). After 5+ min, some nuclei lost fluorescence, which concentrated in small rod to ovid-shaped collections that migrated toward the plasma membrane. We interpreted this to represent an agonal event related to the un-physiologic conditions.*

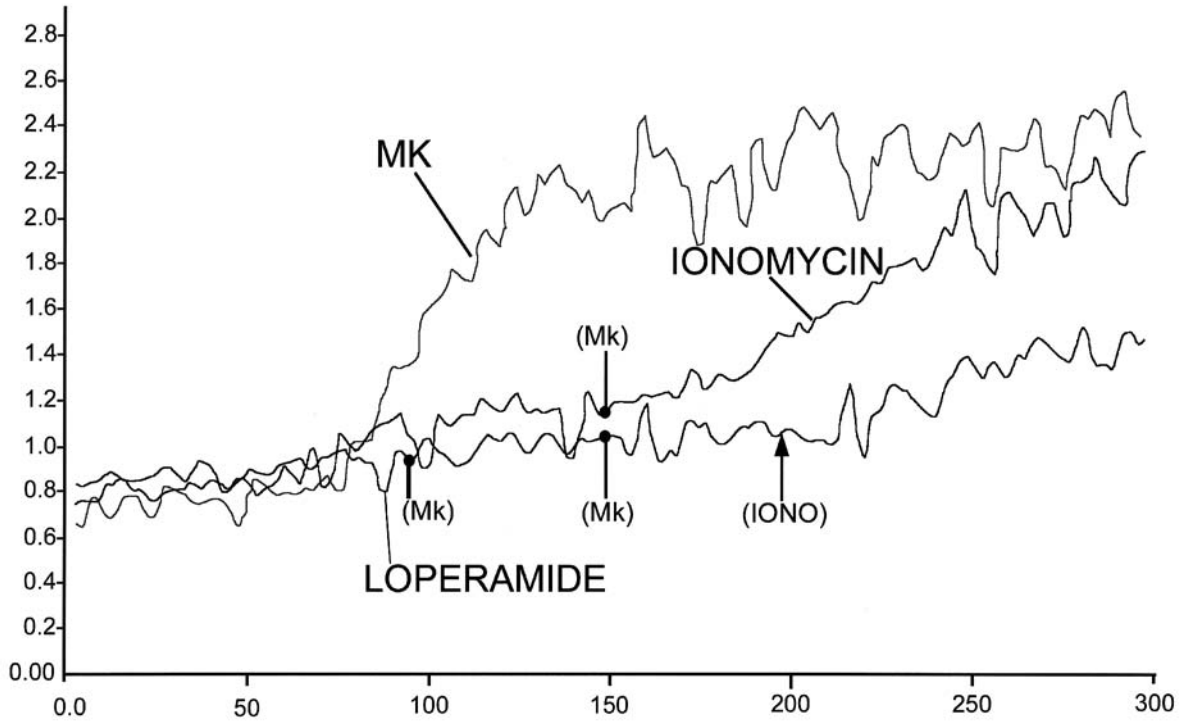

Figure 2. *Representative response of Bcl-2-positive HeLa cell Ca^{2+} to MK 886, assessed after loading with Fura-2 in Ca^{2+} and Mg^{2+}-free Hank's buffer (9). Abscissa, seconds, ordinate R values reflecting changes in fluorescence ration. Ionomycin (10 mM) at 50 seconds, followed by MK 886 (40 uM) denoted by arrows. Loperamide, 10 mM at 50 seconds, followed by Mk 886 at 100 and 150 seconds, and ionomycin at 200 seconds.*

markedly, exhibiting much more variability than cells loaded with Fura-2 or Rhod-2. Collateral evidence for an induced oxidative stress was provided by a study employing a Clontech cDNA "chip" with semi-synchronized Panc-1 cells cultured with MK 886 for 24 h in which increased mRNAs for several heat shock proteins, cytochrome p450 and glutathione reductase, consistent with a response to increased ROS occurred (10). Parenthetically, the other point of interest concerned the large number of mRNAs related to the cell cycle, stress-related DNA synthesis and repair, presumably conferring a countervailing survival advantage to those cells undergoing "chemotherapeutic" challenge.

Table III includes a summary of some properties and responses of the 3 cell lines.

Agents that did not alter the acutely induced MK 886 U937 Ca^{2+} signal. Table IV includes results with a variety of agents examined for their potential ability to alter an acute effect of MK 886 on the release of Ca^{2+} (7, 8). Agents potentially able to alter oxidative stress, Bcl-2 function, a redox inhibitor, a Ca^{2+} ionophore, an L-channel blocker, several inhibitors of mitochondrial function, modulators of cyclic AMP and enzymes unable to enter cells that might exert effects externally were surveyed. As expected, ionomycin as a positive control did increase Ca^{2+}.

Involvement of mitochondria in these events. In U937, Panc-1 and PC3 cells, MK 886 caused a loss of fluorescence in cells preloaded with the mitochondrial dye, R123 (2). This is considered a response to a fall in mitochondrial membrane potential, which can proceed to a mitochondrial membrane permeability transition and opening of membrane "pores" (11). As all 3 cell lines exhibited an MK 886-induced increase in ROS, these two features alone can not be determinative for the form of PCD or the increased U937 nuclear Ca^{2+}.

MK 886 inhibits cellular oxygen consumption and ATP synthesis, resulting in a form of anoxic stress (12). In some circumstances the agent may serve as an ion-transport molecule, although this conclusion was not unequivocal *e.g.*, lack of Ca^{2+} response in PC3 and Panc-1 cells. The reduced internal release and relocation of U937 Ca^{2+} due to inhibitors of mitochondrial function with dissimilar mechanisms of action, including atractyloside, antimycin A and cyclosporin A, are difficult to rationalize (see below). These agents can produce concentration-dependent antagonistic effects on a number of mitochondrial functions, including agonistic (atractyloside) or inhibitory responses (antimycin A and cyclosporin A) on mitochondrial membrane potential and opening of membrane "pores" (13-15; Figure 3A).

Table III. *Comparative characteristics of and responses to 5-LPox inhibitors by U937, Panc-1 and PC3 cells (2,4,5,8,9).*

	U937		Panc-1		PC3	
5-LPox mRNA	+		0		+	
FLAP mRNA	+		+		+	
Bcl-2 protein	+		0		0	
Ca^{2+} response to inhibitors	+		0		0	
Anti-prolif response	+		+		+	
Other Bcl-2 mRNAs	+		+		+	
Inhibition of Bcl-2/ family mRNAs?	SC <	MK <	SC <	MK <	SC <	MK <
Programmed cell death						
Type 1	a,b,c,d	a,b,c,d	-----	-------	----	-----
Atypical#1	----	----	a,b,c,d	----	a,b,c,d	----
Type 2	---	---	----	----	----	a,b,c,d
Atypical #2	---	---	---	a.b.c.d	----	----

Supporting evidence: a, flow cytometry; b, TUNEL; c, DNA laddering; d, morphology.
+, present; 0, absent; , <, Reduced.

Other agents that inhibit the U937 nuclear Ca^{2+} "signal". Loperamide, an L/N ion channel inhibitor, prevented the rise of Ca^{2+}, as did 2 inhibitors of Bcl-2 function, HA-14 (16) and 2-methoxyantimycin A_3 (17), the latter two agents after incubation with U937 cells at 37˚C. This provides suggestive evidence for participation of Bcl-2 in the MK 886-induced rise in Ca^{2+} and involvement of an ion channel (Figure 3A). However, both agents exert multiple effects on cells; for example, HA-14 can induce PCD.

MK 886 induces caspase activity in U937 cells but reduces its expression in actinomycin D-treated Panc-1 cells. A Caspa Tag fluorescence caspase assay (Intergen, Pardonsville, NY, USA) globally detecting caspases 1 through 9, was activated in MK 886-treated U937 cells (work in progress). Actinomycin D increased caspase activity in Panc-1 cells as a component of a type 1 response, but MK 886 reduced the increase (5). The reason for this inhibition is not established but it does not seem to have been due to direct reduction in Panc-1 caspase enzyme activity by ROS or by MK 886, since U937 cells exposed to MK 886 express caspase activity. When Panc-1 cells were cultured with both agents, cell death was incompletely additive (5). However, increased numbers of necrotic cells were present, implying that some identical target cells were affected by simultaneous exposure to both agents, which could contribute to reduced caspase activity. Yet this was not seen with MK 886-incubated U937 cells. Furthermore, the number of surviving Pan-1 "stem" cells, assessed by colony counts, were increased in simultaneously cultured preparations.

Table IV. *Acute effects of agents of interest on MK 886-induced Ca^{2+} (9).*

Any changes in fluorescence were recorded after exposure to the agents of interest for 50 seconds, followed by MK 886 to a final concentration of 40 μM. In companion experiments, increases in Ca^{2+} due to MK 886 alone was followed by addition of agents of interest, in order to detect any quenching.

Agents excluded from cells that detoxify ROS.	**Concentration**
superoxide dismutase	3000 U / ml
catalase	200 U / ml
peroxidase	5000 U / ml
Agents that acutely inhibited or prevented the rise in Ca^{2+}	
antimycin A (inhibits e- transfer at complex III)	10 μM
atractyloside(inhibits ADP/ATP, release cytoC)	25 μM
loperamide (Ca^{2+} L channel inhibitor)	10 μM
cyclosporin A (inhibits phos/tase 2B and nitric ox syn)	20 nM
N-acetyl-cysteine(antioxidant)	50 μM
Agents without acute effects on Ca^{2+}	
H_2O_2 (oxidizing agent)	0.1%
NTBN (free radical spin trap)	100 μM
butylated hydroxy-toluene	100 μM
mercapto ethanol	100 μM
dithiothreitol	100 μM
sodium arsenate (uncouples oxidative phos)	100 μM
sodium cyanide(inhibits terminal e- transport)	2 μM
nifedipine(Ca^{2+} channel blocker, 1,4r dihydriopyridine type)	5 μM
ionomycin (Ca^{2+} ionophore)	5 μM
thapsigargin (inhibits E.R. ATPase, IP3 Ca^{2+} release)	10 nM
2-methyl-antimycin A3(Bcl-2 inhibitor)	10 nM
wortmannin (IP3 kinase inhibitor)	50 μM
SC 41661A (5-lipoxy inhibitor)	40 μM
theophyllin (phiosphodiesterase inhibitor	100 μM
caffeine (cAMP phosphodiesterase inhibitor	100μM
indomethacin (cyclooxygenase inhibitor)	100 μM
Agents reported to inhibit Bcl-2 function that reduced the Ca^{2+} response following prior incubation with cells at 37˚C	
HA 14-1	24 μM
2-methyl-antimycin A_3	5 μM

If an explanation based on increased necrotic cell death following combined culture with both agents does not fully account for these observations, the development of peptidomimetic agents for activating the intrinsic or extrinsic PCD pathways provides further suggestions regarding MK 886-dependent interference with caspase activity in actinomycin D-treated Panc-1 cells, considered subsequently (18,19).

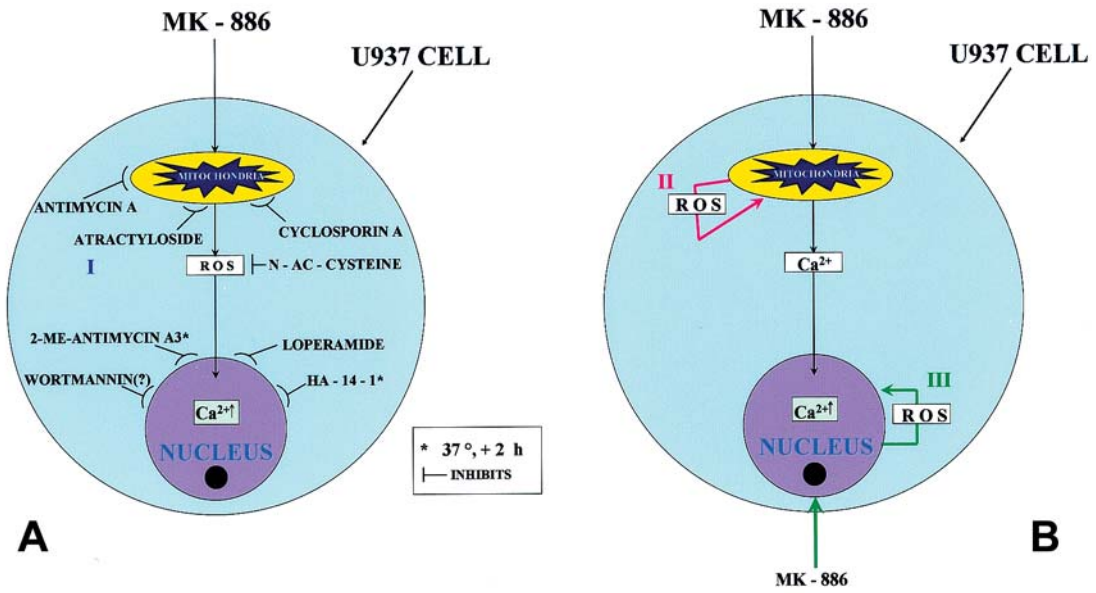

Figure 3A,B. *One proposed major pathway (I) thought to contribute to the acute release of Ca^{2+} from U937 nuclear sites by MK 886 (3A; I) and 2 additional pathways (3B; II and III), that may also participate are depicted (9). Reactive oxygen (or nitrogen) species generated in mitochondria or at other intracellular sites that act either locally (II, possibly III) or more distantly (I), depending upon their half lives and another circumstances, release, possibly with involvement of Bcl-2 and a Ca^{2+} ion channel of Ca^{2+} at its site(s) or origin, (II, III) or more distantly, which can diffuse to and be detected at sites containing resident Fura-2. Some of these events may occur asynchronously in the same or in different cells.*

Discussion

The probable site of ROS formation. There seems to be general agreement that mitochondria represent a major site of ROS formation (20). Most, although possibly not all, of the DCDHF fluorescence in MK886-treated U937 cells remained associated with mitochondria and adjacent endoplasmic reticulum. Rat liver nuclei exhibit a limited form of respiration with oxidative phosphorylation and the formation of ROS (21). Studies with DCDHF provided little evidence of an MK 886-induced increase in U937 nucleus-associated ROS. While the question remains open, the extent to which nuclei may generate ROS from respiration appears to be limited.

The sub-cellular source of Ca^{2+}. After exposure to MK 886, U937 cytoplasmic Rhod-2 fluorescence did not alter significantly, suggesting that mitochondria, a major storage site for calcium (22-24), and the "non-nuclear" endoplasmic reticulum (25), were not primarily responsible for a release of Ca^{2+}. Attention has been directed to peri-nuclear endoplasmic reticulum and nuclear sources.

There is evidence that nuclear Ca^{2+} can originate from cytosol, juxta-nuclear and nuclear sources, including the endoplasmic reticulum in continuity with components of the nuclear envelope and its extensions into the nucleoplasm (26-28). The cyclic AMP-response element, CRE and the CREB binding protein in ATt20 cells function as a response element sensitive to nuclear Ca^{2+}-dependent transcription, distinct from transcription due to the serum response element (SRE) sensitive to cytoplasmic Ca^{2+} (29,30).

A role for Bcl-2? There is a prima facie case for a Ca^{2+} channel associated with Bcl-2 that may be juxta-nuclear or nuclear in location. As mentioned, 40 μM MK 886 added to U937 cells pre-loaded with Fura-2 requires only 10-20 sec to increase cellular fluorescence that is primarily nuclear in location. To demonstrate the suggested relation between MK 886-induced ROS, Bcl-2 protein, a nuclear Ca^{2+} signal and a form of type 1 PCD will require the suppression of Bcl-2 mRNA, decline in the Bcl-2 protein and loss or attenuation of the MK 886-induced nuclear Ca^{2+} signal. Until such experiments are completed, a relationship based on the available, admittedly circumstantial evidence is suggested (Figure 3B, I).

Do ROS release Ca^{2+} locally or distantly? Provisionally, we suggest that MK 886 increases ROS formation, probably by mitochondria, the major cellular source of ROS (and of reactive nitrogen species), the effects of which can be abrogated by the antioxidant, N-acetyl-L-cysteine. An increase in ROS and their diffusion is followed by modification,

perhaps through a conformational change of a Bcl-2 (or immunologically-related) protein associated with an ion channel (31, 32) mediating Ca^{2+} that is in close proximity with the peri-nuclear endoplasmic reticulum and conjoined nuclear membrane or even within nuclei and their nucleoplasmic reticulum (26-28). The local increase in ROS modifies the Ca^{2+} storage site, perhaps releasing the ion to a Bcl-2-related channel-associated protein by altering the oxidation / reduction potential at one or more sites. (Figure 3B.). ROS probably diffuse from regions remote from nuclei or, less probably, might be generated at nuclear loci (Fig 3B, III). It is unclear if the inhibitors of mitochondrial function employed would interfere with any putative nuclear oxidative phosphorylation and local generation of ROS.

It should be mentioned that, in an earlier study employing cultured prostate cells, it was demonstrated that in Bcl-2 over-expressing cells, Ca^{2+} uptake by isolated nuclei was reduced compared with controls. (33).

Effects of mitochondrial inhibitors on the U937 Ca^{2+} signal. Neither the role of mitochondria in the provision of U937 nuclear Ca^{2+}, nor their response to some but not other inhibitors of mitochondria, are well defined. A parsimonious explanation for the latter could include reduced formation or release of ROS by the active inhibitors but not the ineffective ones, a point to be examined. Although mitochondria represent a major site of Ca^{2+} retention, the studies with Rhod-2 provided no clear evidence for a major release of Ca^{2+} from mitochondria as the primary source of the increased nuclear Fura-2 fluorescence. In fact, mitochondrial uptake of Ca^{2+} may be more likely (34).

Acute exposure of U937 cells to antimycin A or atractyloside, which inhibit specific steps of oxidative phosphorylation that can have opposite effects on MMP, or to cyclosporin A which closes mitochondrial "pores", all reduced or prevented MK 886-induced rise in Ca^{2+} without themselves generating a signal detected by Fura 2.

How to rationalize the difference in response to MK 886 of the hematopoietic and solid cancer cells examined to date? U937 cells developed a classic apoptotic response to MK 886, while Panc 1 and PC3 cells, exhibiting a type 1 response to actinomycin D or SC41661A, respectively, expressed a "type 2" non-apoptotic form of cell death. Cells of either provenance retain an ability to express both forms, at least to some extent, given an agent with which they can interact (35; HL-60 cells and caspase-negative cell death). In view of the major role of caspases in the implementation of type 1 PCD, one basic deficiency in Panc-1 and PC3 cell response to MK 886 appears to their less robust or apparently absent activation of caspase enzymes, potentially including 1 through 9, assessed with a Caspa Tag kit. Activation of an endoplasmic reticulum-related stress response involving increased caspase 12 (36) or other forms of a "stress-induced" type 2 PCD (37,38) might represent default responses, perhaps of a more ancient evolutionary origin (39). Of course these comments assume a simple explanation of greater necrotic cell killing in combined culture, which however was not reflected in any significantly increased cell killing and may not explain the reduced caspase activity.

Caspases are considered to be activated either by oligomerization or by initiator or effector caspases. Blocking BID-induced BAK and BAX-related cytochrome C release leading to the formation of the APAF-1 / caspase 9 "apoptosome" by an MK 886-related activity formally could account for MK 886-related Panc-1 caspase enzyme inhibition (19). The extent of potential caspase 1 through 9 activity in MK 886-treated U937 cells might depend upon activity of components associated with an extrinsic pathway (20), absent or under-represented in Panc-1 or PC3 cells. For example, Panc-1 cells lack an apoptotic-responsive functional FAS ligand (CD95/APO 1) signaling system (40). Lack of caspase 3 activity renders leukemic cells resistant to induction of apoptosis (41). The relative inability of MK 886 to inhibit Bcl-2-family- related mRNA synthesis in Panc-1 and PC3 cells, compared with its greater suppression in U937 cells, may contribute in some currently obscure manner (2).

Possibly differential expression of oxidant-related or redox-initiated events contributes to these differences (42). An interplay between activation of protein kinases and inhibition of protein phosphatases by oxidants and their reciprocal responses to antioxidants alludes to potential interactions in cells of different lineages (43). Hydrogen peroxide-activation of AP-1 involving increased Ca^{2+}, activation of tyrosine and other kinases and their inhibition by antioxidants is paradigmatic of such interactions. Calcium ion activation of proteolysis and the role of IP3 in its release provides another avenue for lineage-specific responses to agents of interest (44).

What are some likely consequences of an early Ca^{2+} "signal" in U937 nuclei? Increased nuclear Ca^{2+}, originating either from contiguous or more remote Ca^{2+} stores, can be expected to alter the activity of various nucleus-associated kinases (PKC, cAMP-dependent) or phosphatases (phosphatase 2B or calcineurin) activities with modulation of transcription factors (as NF-AT) and the synthesis of early mRNAs en train (45,46). Whether any of these putative events contribute to the selection of a "type 1" apoptotic rather than a "type 2" non-apoptotic PCD is a further issue. As mentioned, the classic inhibitor of RNA synthesis, actinomycin D, induces a type 1 PCD in Panc-1 cells (6), while MK 886, which inhibits the Bcl-2 family mRNA synthesis in U937 but less extensively in Panc-1 and PC3 cells, leads in these cells to type 1 and type 2 PCD, respectively.

Some implications of a differential expression of several types of PCD; are their effects mutually synergistic, additive, null or inhibitory? In Panc-1 cells the combined induction of both forms due to simultaneous culture with actinomycin D and MK 886 was associated with increased necrotic cell death, accompanied by contributions from types 1 and 2 forms of programmed death (5). However, when colony counts were studied, more surviving colonies were obtained from the "combined" cultures compared with either agent alone (5; but see ref. 47 for a contrary result). The former result suggests that the replicating "targets" for these agents, alone or in combination, differed. There has been controversy about whether the appropriate measure of cancer cell response to therapy is destruction of the bulk of cells, especially as reflected by their apoptosis (48), most of which may have been incapable of further cell division, or of a more fundamental population of replicating progeny cells identified as a "stem" cell population (49-51). Not much is know about the interplay between different forms of cell death; programmed, non-replicative, necrotic forms or due to immunological responses as they develop in different cell populations, especially in response to multiple agents (39). Examples of cross-talk between extrinsic and intrinsic PCD pathways *via* the pro-apoptotic protein BID have been reported, *e.g.*, ref. 52.

Can restrictions inherent in a "soil" versus "seed" mismatch be circumvented? Unless cancer cells of interest, in particular members of a putative "stem" cell population, interact with an agent *via* a receptor, ion channel, enzyme or other component, no response should be expected. Generally the focus has been to identify molecules inhibiting signal transduction; activating a therapeutic response by a biologic sleight-of-hand appears to present more difficulties.

Global genomic or protonomic studies of bulk or of micro-dissected cancer cell preparations have been useful in providing prognostic information and identifying signaling pathways and molecules that correlate with a malignantly transformed biology. Identifying events responsible for the transformed behavior of most cancer cells should seem to require first identifying a defect in the purported "stem" cells. The expression in chronic myelogenous leukemia cells of the BCR-ABL mutation with aberrant tyrosine kinase activity demonstrates this requirement for an identifiable biochemical "tag" necessary for the subsequent development of STI 571 (Gleevec, Imatinib), able initially to suppress that enzyme. Development of BCR-ABL protein point-mutations conferring resistance to ST571 have led to second generation compounds able to inhibit growth of bone marrow progenitors from resistant cells (53). Reports of cancer cell aberrations in which only a mutated, activated receptor such as EGFR in non-small lung cancer cells responds to an agent such as Genfitinib (Iressa or ZD 1839), while broadening the complexity of potential therapeutic approaches, provides a sought-after specificity of response (54). Further speculation in this venue is probably unwarranted.

A necessary caveat. It is understood that observations obtained with these agents would probably be different had higher concentrations and longer exposure under more physiologic conditions, *e.g.*, Ca^{2+} -containing buffer etc., been employed. Exposure was brief, lasting 30 seconds before challenge with 40 μM MK 886. Most of the agents employed exert multiple concentration- and time-dependent effects on cells, some of which may be lineage-dependent. The end point of interest, increased nuclear Fura-2 fluorescence, only became evident after 10 or more seconds. Numerous events in various regions of different populations of cells may have occurred prior to this, some in a millisecond time-frame at an intensity not readily detected by the unaided eye, which might have been identified with a more searching kinetic analysis of multiple cells. Consequently, several mechanistic options are suggested in Figure 3 A and B. Contributions to nuclear Ca^{2+} from multiple sources leading to discrete consequences would not surprise.

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References

1 Anderson KM, Seed T, Jajeh A, Dudeja P, Byun T, Meng J, Ou D, Bonomi P and Harris JE: An *in vivo* inhibitor of 5-lipoxygenase, MK886, at macromolar concentration induces apoptosis in U937 and CML cells. Anticancer Res *16*: 2589-2600, 1996.

2 Anderson KM, Alrefai WA, Dudeja PK, Jadko S, Bonomi P, Hu Y and Harris JE: Increased cytosol Ca^{2+} and type 1 programmed cell death in Bcl-2-positive U937 but not in Bcl-2-negative PC3 and Panc-1 cells induced by the 5-lipoxygenase inhibitor MK 886. Prost Leuko Ess Fatty Acids *66*: 443-452, 2002.

3 Dittmann KH, Mayer C, Rodemann HP, Petrides PE and Danzlinger C: MK-886, aleukotriene biosynthetic inhibitor, induces antiproliferative effects and apoptosis in HL-60 cells. Leuk Res *22*: 49-53, 1998.

4 Anderson KM, Seed T, Vos M, Mulshine J, Meng J, Alrefai W, Ou D and Harris JE: Five-lipoxygenase inhibitors reduce PC3 cell proliferation and initiate non-necrotic cell death. The Prostate *37*: 161-173, 1998.

5 Anderson KM, Alrefai WA, Bonomi P, Seed T, Dudeja PK, Hu Y and Harris JE: Caspase-dependent and independent Panc-1 cell death due to actinomycin D and MK 886 are additive but increase clonogenic survival. Exp Biol Med *228*: 399-407, 2003.

6 Kleeff J, Kornmann M, Sawhney H and Korc M: Actinomycin D induced apoptosis and inhibits growth of pancreatic cancer cells. Int J Cancer *86*: 399-407, 2000.

7 Arefai WA, Anderson KM, Bonomi P, Hu Y, Ou D and Harris JE: Acute changes in U937 nuclear Ca^{2+} induced to undergo programmed cell death by MK 886. Proc Am Assoc Cancer Res *44*; (#5037), 1155, 2003.

8 Anderson KM, Rubenstein M, Alrefai WA, Dudeja P, Tsui P, Guinan P and Harris JE: Acute changes in U937 nuclear Ca^{2+} preceding type 1 apoptotic programmed cell death due to MK 886. Anticancer Res, *24*: 2001-2016, 2004.

9 Anderson KM, Rubenstein M, Tsui P and Harris JE: Disparate forms of MK-886-induced programmed cell death in Bcl-2 (+) blood and Bcl-2 (-) solid cancer cells and a putative "nuclear" Ca^{2+} channel: "soil" trumps "seed". Med Hypo, in press, 2004.

10 Anderson KM, Alrefai W, Anderson C, Bonomi P and Harris J: MK 886 functions as a radiomimetic agent: genomic responses related to oxidative stress, the cell cycle, proliferation and programmed cell death in Panc-1 cells. *In*: Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury, 5, ed Honn *et al*., Kluwer Academic/Plenum Pub, 2002, pp 451-456.

11 Kowaltowski AJ, Castilho RF and Vercesi AE: Mitochondrial permeability transition and oxidative stress. FEBS Lett *495*: 12-15, 2001.

12 Gugliucci A, Ranzato L, Scorrano L, Colonna R, Petronilli V, Cusan C, Prato M, Mancini M, Pagano F and Bernardi P: Mitochondria are direct targets of the lipoxygenase inhibitor MK 886. JBC *277*: 31789-31795, 2002.

13 Giodarelli A, Fiorani M and Cantoni D: Calcium-dependent mitochondrial formation of species promoting strand scission of genomic DNA in U937 cells exposed to tert-butyl hydroperoxide: the role of arachidonic acid. Free Rad Res *33*: 477-487, 2000.

14 Byrne AM, Lemaster JJ and Nieminen AL: Contribution of increased mitochondrial free Ca^{2+} to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes. Hepatology *29*: 1523-1531, 1999.

15 First DJ, Lynch RC and Leiu RW: Cyclosporin A inhibits nitric oxide production by L-929 cells in response to tumor necrosis factor and interferon gamma. J Inter Res *13*: 235-240. 1993.

16 Wang J-L, Liu D, Zhang Z-J, Shan S, Han X, Srinivasa SM, Croce CM, Alnemri ES and Huang Z: Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. PNAS US *97*: 7124-7129, 2000.

17 Tzung S-P, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, Zhang KYJ and Hockenberry DM: Antimycin A mimics a cell death-inducing BCL-2 homology domain 3. Nat Cell Biol *3*: 185-191, 2004.

18 Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL and Korsmeyer SJ: Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. Science *305*: 1466-1470, 2004.

19 Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X and Harran PG: A small molecular Smac mimic potentiates TRAIL and TNFa-mediated cell death. Science *305*: 1471-1474, 2004.

20 Turens JF: Mitochondrial formation of reactive oxygen species. J Physiol *552.2*: 335-344, 2003.

21 Kukieka E and Cederbaum AI: NADPH and NADP-dependent oxygen radical generation by rat liver nuclei in the presence of redox cycling agents and iron. Arch Biochem Biophys *283*: 326-333, 1990.

22 Hoth M, Fanger CM and Lewis RS: Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol *137*: 633-648, 1997.

23 Bowser DN, Petrou S, Panchal RG, Smart ML and Williams PA: Release of mitochondrial Ca^{2+} permeability transition activates endoplasmic reticular Ca^{2+} uptake. FASEB J *16*: 1105-1107, 2002.

24 Stout AK, Raphael HM, Kanterewicz BI, Klarin E and Reynolds IJ: Glutamate-induced neuron death requires calcium uptake. Neurosci *1*: 366-378, 1998.

25 Tribe RM, Borin ML and Blaustein MP: Functionally and spatially distinct Ca^{2+} stores are revealed in cultured vascular smooth muscle cells. PNAS US *91*: 5908-5912, 1994.

26 Allbritton NL, Oancea E, Kuhn MA and Meyer T: Source of nuclear calcium signals. PNAS US *91*: 12458-12462, 1994.

27 Santella L and Carafoli E: Calcium signaling in the nucleus. FASEB J *11*: 1091-1109, 1997.

28 Echevarri W, Leite MF, Guerra MT, Zipfel WR and Nathanson MH: Regulation of calcium signals in the nucleus by a nucleoplasmic reticulum. Nat Cell Biol *5*: 440- 446, 2003.

29 Hardingham GE, Chawla S, Johnson CM and Bading H: Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. Nature *385*: 260-265, 1997.

30 Rogue PJ and Malviya AN: Calcium signals in the cell nucleus. EMBO J *18*: 5147-5152, 1999.

31 Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M and Reed JC: Channel formation by antiapoptotic protein Bcl-2. PNAS US *94*: 5113-5118, 1997.

32 Vieira HL, Boya P, Cohen I, Hamel EL, Haouzi D, Druillenec S, Belzacq AS, Brenner C, Rogues B and Kroemer G: Cell permeable BH-3 peptides overcome the cytoprotective effect of Bcl-2 and Bcl-X(L). Oncogene *21*: 1963-1977, 2002.

33 Marin A, Bick MC, Fernandez RJ, Brisbay S, Buja LM, Snuggs M, McConkey DJ, von Eschenback AC, Keating MJ and McDonnell TJ: Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca^{2+}. Oncogene *12*: 2259-2266, 1996.

34 Murphy AN, Bredesen DE, Cortopassi G, Wang E and Friskum G: Bcl-2 potentiates the maximal calcium uptake capacity of neurol cell mitochondria. PNAS US *93*: 9893-9898, 1996.

35 Lecureur V, Lagadic-Gossmann D and Fardidl O: Potassium antimonyl tartrate induces reactive oxygen species-related apoptosis in human myeloid cells. Int J Oncol *20*: 1071-1076, 2002.

36 Nakagawa T, Zhu H, Motrrrishime N, Li E, Xu J, Yankner BE and Yuan J: Caspase 12 mediates endoplasmic-related specific apoptosis and cytotoxicity of amyloid-beta. Nature *403*: 98-103, 2000.

37 Herrera B, Alvarez AM, Sanchez A, Fernandez M, Roncero C, Benito M and Fabregat I: Reactive oxygen species (ROS) mediate the mitochondrial-dependent apoptosis induced by transforming growth factor B in fetal hepatocytes. FASEB J *15*: 741-751, 2001.

38 Bursch W: The autosomal-lysosomal compartment in programmed cell death. Cell Death Diff *8*: 569-581, 2001.

39 Yin X-M and Dong Z: Essentials of Apoptosis. Human Press, NJ, 2003, pp 102-117.

40 Ungerforen H, Voss M, Jansen M, Roeder C, Hdenne-Bruins D, Kroemer R and Kaltholff H: Human pancreatic adenocarcinoma express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis. Cancer Res *68*: 1741-1749, 1998.

41 Choi Y-J, Park J-W, Woo J-H, Kim Y-H, Lee S-H, Lee J-M and Kwon TK: Failure to activate caspase 3 in phorbol ester-resistant leukemia cells is associated with resistance to apoptotic cell death. Cancer Lett *182*: 183-191, 2002.

42 Anderson MT, Staal FJT, Gitler C, Herzenberg LA and Herzenberg LA: Separation of oxidant-initiated and redox-regulated steps in NF-Î B signal transduction pathway. PNAS US *91*: 11527-11531, 1994.

43 Sen CK and Packer L: Antioxidant and redox regulation of gene transcription. FASEB J *110*: 709-720, 1996.

44 Szalai G, Krishnamurthy R and Hajnoczky G: Apoptosis driven by IP3-linked mitochondrial calcium signals. EMBO J *18*: 6349-6361, 1999.

45 Feske S, Giltnane J, Dolmetsch R, Staudt LM and Rao A: Gene regulation mediated by calcium signals in T lymphocytes. Nat Immunol *2*: 316-324, 2001.

46 Chaung Y-YE, Chen Y, Godisetti C, Chandramaoul Sludla I VR, Cook JA, Coffin D, Tsai M-H, DeGraff W, Yan H, Zhao S, Ruassa A, Liu ET and Mitchell JB: Gene expression after treatment with hydrogen peroxide, menadione or t-butyl hydroperoxide in breast cancer cells. Cancer Res *62*: 6246-6254, 2002.

47 Lock RB and Stribinskiene L: Dual modes of death by ectopopside in human epithelial tumor cells allows Bcl-2 to inhibit apoptosis without affecting clonogenic survival. Cancer Res *56*: 4006-4012, 1996.

48 Tannock IF and Lee C: Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anticancer drugs. Br J Cancer *84*: 100-105, 2001.

49 Li L and Akashi K: Unraveling the molecular components and genetic blueprints of stem cells. Biotechniques *35*: 1233-1239, 2003.

50 Pazianos G, Ugoezwa M and Reya T: The elements of stem cell self-renewal: a genetic perspective. Biotechniques *35*: 1240-12249, 2003.

51 Jamieson CHM, Ailles LE, Dylla SJ *et al*: Granulocytic-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. New Eng J Med *351*: 6576-671, 2004.

52 Gross A, Yin X-M, Wang K *et al*: Caspase cleaved BID targets mitochondria and is required for cytochrome c release while BCL-XL prevents their release but not TNF-R1/Fas death. JBC *271*: 1156-1163, 1999.

53 Shah NP, Tran C, Lee FY, Chen P, Norris D and Sawyers CL: Overriding imatinib resistance with a novel ABL kinase inhibitor. Science *305*: 399-401, 2004.

54 Tracy S, Mukohara T, Hanasen M, Meyerson M, Johnson BE and Jenne PA: Genfitinib induces apoptosis in non small-cell lung cancer cell line H3255. Cancer Res *64*: 7241-7244, 2004.

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