Tipifarnib-Induced Apoptosis in Acute Myeloid Leukemia and Multiple Myeloma Cells Depends on Ca\(^{2+}\) Influx through Plasma Membrane Ca\(^{2+}\) Channels\(^{[S]}\)

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ABSTRACT

A major contributing factor to the high mortality rate associated with acute myeloid leukemia and multiple myeloma is the development of resistance to chemotherapy. We have shown that the combination of tipifarnib, a nonpeptidomimetic farnesyltransferase inhibitor (FTI), with bortezomib, a proteasome inhibitor, promotes synergistic death and overcomes de novo drug resistance in acute myeloid leukemia cell lines. Experiments were undertaken to identify the molecular mechanisms by which tipifarnib produces cell death in acute myeloid leukemia and multiple myeloma cell lines (U937 and 8226, respectively). Tipifarnib, but not other FTIs tested [N-[4-[2-(R)-amino-3-mercaptopyrrol-2-phenylbenzoyl]methionine methyl ester trifluoroacetate salt (FTI-277) and 2-methyl-5-[[1-trityl-1H-imidazol-4-yl]yl]-1-piperidinyl]-2-oxoethyl]-1-piperidinecarboxamide, promotes elevations in intracellular free-calcium concentrations ([Ca\(^{2+}\)\(_{i}\)]) in both cell lines. These elevations in [Ca\(^{2+}\)\(_{i}\)] were accompanied by highly dynamic plasmalemmal blebbing and frequently resulted in membrane lysis. The tipifarnib-induced elevations in [Ca\(^{2+}\)\(_{j}\)], were not blocked by thapsigargin or ruthenium red, but were inhibited by application of Ca\(^{2+}\)-free extracellular solution and by the Ca\(^{2+}\) channel blockers Gd\(^{3+}\) and La\(^{3+}\). Conversely, 2-aminoxydihydrophenyl borate (2-APB) potentiated the tipifarnib-evoked [Ca\(^{2+}\)\(_{j}\)] overload. Preventing Ca\(^{2+}\) influx diminished tipifarnib-evoked cell death, whereas 2-APB potentiated this effect, demonstrating a link between tipifarnib-induced Ca\(^{2+}\) influx and apoptosis. These data suggest that tipifarnib exerts its effects by acting on a membrane channel with pharmacological properties consistent with store-operated channels containing the Orai3 subunit. It is noteworthy that Orai3 transcripts were found to be expressed at lower levels in tipifarnib-resistant 8226/R5 cells. Our results indicate tipifarnib causes cell death via a novel mechanism involving activation of a plasma membrane Ca\(^{2+}\) channel and intracellular Ca\(^{2+}\) overload.

Introduction

Farnesyltransferase inhibitors (FTIs) are a novel class of anticancer agents developed to inhibit the enzyme farnesyltransferase that is responsible for the transfer of a farnesyl group to the Ras protein. FTIs were originally designed to inhibit Ras oncogenic activity, but studies have suggested that FTIs may have several other targets, including centromeric proteins and the phosphatidylinositol-3 kinase/Akt pathway (Ashar et al., 2000; Jiang et al., 2000). To date, several FTIs have been clinically evaluated, including...
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The mitochondrial Ca²⁺ dria via a mechanism that seems to depend on the reversal of store-operated channel (SOC) subunit Orai3.

Tipifarnib-induced disruption of Ca²⁺ mitochondria- and ER-independent manner in these cells.

line models. We show that tipifarnib dysregulates Ca²⁺ myeloid leukemia (U937) and a multiple myeloma (8226) cell synergistically with bortezomib or the FTI is targeting a via an identical mechanism to induce cell death and act.

However, it remains to be determined whether tipifarnib acts stress in myeloma cells by inducing Ca²⁺

The adhesion of tumor cells to fibronectin and bone marrow stromal cells has been implicated in the cellular rearrangement of molecules involved in drug resistance including caspase 8 homologue FLICE-inhibitory protein, topoisomerase IIB, Fas, and Bcl-2 (Hazelhurst et al., 2001). This particular form of de novo drug resistance is called cell adhesion-mediated drug resistance (CAM-DR). Previously, we have shown that the combination of the farnesyltransferase inhibitor, tipifarnib, and proteasome inhibitor, bortezomib, induces the ER stress response synergistically in myeloma cells and overcomes CAM-DR (Yanamandra et al., 2006). Bortezomib, as a single agent, dysregulates [Ca²⁺]i, and elicits ER stress in myeloma cells by inducing Ca²⁺ from the mitochondria via a mechanism that seems to depend on the reversal of the mitochondrial Ca²⁺ uniporter (Landowski et al., 2005). However, it remains to be determined whether tipifarnib acts via an identical mechanism to induce cell death and act synergistically with bortezomib or the FTI is targeting a second molecular mechanism that also culminates in the induction of ER stress and concomitant cell death.

In the present study we examined the mechanisms by which tipifarnib induces ER stress and apoptosis in acute myeloid leukemia (U937) and a multiple myeloma (8226) cell line models. We show that tipifarnib dysregulates [Ca²⁺]i in a mitochondria- and ER-independent manner in these cells. Tipifarnib-induced disruption of [Ca²⁺]i, homeostasis and apoptosis was found to involve activation of a plasma membrane Ca²⁺ channel and correlated with expression of the store-operated channel (SOC) subunit Orai3.

Materials and Methods

Cell Lines and Cell Culture. U937 and 8226 cells were obtained from the American Type Culture Collection (Manassas, VA). The 8226/R5 line was developed in the laboratory of Dr. Darrin Beaupre and has been described previously (Buzzeo et al., 2005). Tumor cell lines were adhered to fibronectin overnight at 37°C as described previously (Yanamandra et al., 2006) before all experiments were carried out, unless otherwise indicated.

Cytotoxicity Assays. Cytotoxicity analysis was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay as described previously (Buzzeo et al., 2005). For all cytotoxicity studies cells were exposed to both calcium-modulating agents and tipifarnib simultaneously for 72 h. After 72 h at 37°C, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO) was added to each well, and cells were incubated for an additional 4 h. All experiments were done in triplicate.

Western Blotting. Western blotting was performed as described previously (Beaupre et al., 2003). Antibodies were purchased from the following vendors: caspase-12 (Abcam Inc., Cambridge MA), poly(ADP-ribose) polymerase 1 (PARP1) (Cell Signaling Technology, Danvers, MA), and β-actin (Sigma-Aldrich). In brief, after tipifarnib treatment cells were harvested by centrifugation, washed once with ice-cold phosphate-buffered saline, and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1 mM EGTA, 50 mM Tris-HCL, pH 7.5, 1% NP-40, and 0.5% SDS) containing 0.2 mM phenylmethylsulfonyl fluoride, 56 μg/ml apronin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, 1 mM NaF, and 10 mM Na3P04. Then equal amounts of proteins were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), probed with the indicated antibody, and developed using an enhanced chemiluminescence reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from 2 × 10⁶ log-phase cells using the Qiagen Kit (QIAGEN, Valencia, CA). RT and PCR was done using the Power SYBR Green RNA-to-Ct one-step kit (Applied Biosystems, Foster City, CA) using Quantitect primers against Hs_GAPDH_2_SG and Hs_ORAI3_1_SG (Q0719091 and Q10233910, respectively; QIAGEN). In brief, 4 ng of total RNA was reacted in a 25-μl final volume using 1× final primer concentrations and recommended cycling specifications for SYBR Green on a StepOnePlus Real-Time PCR machine (Applied Biosystems). Reactions were performed in triplicate for target (ORAI3) and endogenous control (glyceraldehyde-3-phosphate dehydrogenase) for each cell line. The experiment was repeated three times independently using freshly isolated RNA. All data were compiled, and relative quantity (RQ) of expression was calculated using the Applied Biosystems algorithm.

Measurement of Intracellular Calcium. Intracellular free calcium was measured using the Ca²⁺-sensitive dye, fura-2, as described previously (DeHaven and Cuevas, 2004). Cells were plated on coverslips coated with poly-lysine (Sigma-Aldrich), which enhances cell adhesion in our model and permits responses to chemotherapeutic agents in leukemia cell lines identical to those obtained with fibronectin (Landowski et al., 2005). Fura-2 loading was carried out incubating the plated cells for 1 h at room temperature in physiological saline solution (PSS) consisting of 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 7.7 mM glucose, and 10 mM HEPES (pH to 7.2 with NaOH), which also contained 1 μM of the membrane-permeable ester form of fura-2, acetoxymethyl ester (fura-2 AM) and 0.1% dimethyl sulfoxide (DMSO). The coverslips were then washed in PSS (fura-2 AM free) before the experiments were carried out. All drugs were bath-applied in PSS.
fit Cancer Center and Research Institute). Calcium-modulating agents, ruthenium red, lanthanum chloride, gadolinium chloride, and 2-APB, were obtained from Sigma-Aldrich, and fura-2 AM was from Invitrogen (Carlsbad, CA).

**Statistical Analysis.** For multiple group comparisons, one-way or two-way analysis of the variance tests were carried out, as appropriate. When the analysis of variance indicated significant difference, a post hoc analysis with Tukey’s test was conducted to identify the group or groups that were significantly different. Unless otherwise stated, statistical data are expressed as mean ± S.E.

**Results**

**Tipifarnib Evokes ER Stress.** We have previously shown that tipifarnib acts synergistically with bortezomib and can overcome CAM-DR in multiple myeloma and acute myeloid leukemia. It has also been suggested that induction of the ER stress response by tipifarnib may be responsible for reversal of the CAM-DR phenotype (Yanamandra et al., 2006). Experiments using U937 cells were conducted to confirm that tipifarnib induces the ER stress response in leukemia cells. Leukemia cells were adhered onto fibronectin to promote CAM-DR and treated with tipifarnib for 48 h. Protein extracts from untreated (control) and tipifarnib-treated U937 cells were probed for expression of procaspase-12, an ER resident caspase that is specifically activated by ER stress (Nakagawa et al., 2000), and PARP, a caspase-3 substrate and the indicator of apoptosis (Oliver et al., 1998).

Figure 1A shows a representative Western blot of protein extracts from control and tipifarnib-treated cells. Tipifarnib induced a dose-dependent decrease in the levels of inactive caspase-12 protein (Fig. 1B). The PARP1 antibody used for our study detects both full-length PARP1 (116 kDa, full length) and a large PARP1 fragment (89 kDa) resulting from caspase cleavage of this peptide. Incubation in tipifarnib resulted in a decrease in full-length PARP1 and a concomitant increase in the 89-kDa fragment (Fig. 1). Likewise, application also resulted in the cleavage of caspase-4, a second caspase implicated in the ER stress response (data not shown) (Hitomi et al., 2004). These data confirm that tipifarnib triggers ER stress-related pathways in adherent leukemia cells.

**Intracellular Calcium Homeostasis Is Dysregulated by Tipifarnib in U937 Leukemia Cells.** ER stress can be induced by various factors, such as disruption of intracellular Ca\(^{2+}\) homeostasis (Rao et al., 2004). We have previously reported that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins.

**Fig. 1.** Tipifarnib promotes ER stress in U937 cells. A, Western blot analysis of protein extracts from U937 cells incubated in normal media (Control) or media containing tipifarnib at the indicated concentrations. Extracts were probed using antibodies directed against cleaved caspase-12, PARP1 (full-length and cleaved), and β-actin, with the latter used as a lane loading reference. B, bar graph of the relative intensity of the bands from A. Data were normalized to the intensity of the β-actin band for each condition. Three regions of interest encompassing each entire band were collected to quantify intensity. PARP1-FL represents the full-length PARP1 protein (upper PARP1 band in A), and PARP1-C represents the 89-kDa caspase cleavage product of PARP1 (lower PARP1 band in A). * indicates significant difference from control, and # indicates significant difference from 1.25 μM tipifarnib (p < 0.01 for all).

**Intracellular Calcium Homeostasis Is Dysregulated by Tipifarnib in U937 Leukemia Cells.** ER stress can be induced by various factors, such as disruption of intracellular Ca\(^{2+}\) homeostasis (Rao et al., 2004). We have previously reported that tipifarnib-resistant 8226 myeloma cells (8226/R5) express high levels of calcium signaling pathway proteins (Buzzeo et al., 2005), raising the possibility that tipifarnib-induced ER stress is also the result of dysregulation of [Ca\(^{2+}\)]\(_i\) homeostasis. The effects of tipifarnib on [Ca\(^{2+}\)]\(_i\) in tumor cells were studied in adhered U937 leukemic cells via Ca\(^{2+}\)\, flurometry using fura-2 as the indicator. Application of 5 μM tipifarnib onto the U937 cells evoked pronounced elevations in [Ca\(^{2+}\)]\(_i\) at concentrations previously shown to have cellular effects (Lerner et al., 1995; Sun et al., 1999) (Fig. 2B). Unlike tipifarnib, the peptidomimetic farnesyl transferase inhibitors FTI-277 and FTI-2153 failed to stimulate elevations in [Ca\(^{2+}\)]\(_i\), at concentrations previously shown to have cellular effects (Lerner et al., 1995; Sun et al., 1999) (Fig. 2B). Membrane lysis, as indicated by a sudden drop in fluorescence, was not observed in any of the cells treated with FTI-277 and FTI-2153. In all cells responding to tipifarnib, the pronounced elevations in [Ca\(^{2+}\)]\(_i\) were accompanied by highly dynamic plasma membrane blebbing (Fig. 2C). This form of membrane blebbing, which has been described as membrane “boiling,” is specifically associated with apoptotic but not necrotic cell death (Laster et al., 1988) and is known to be caused by [Ca\(^{2+}\)]\(_i\) overload (Furuya et al., 1994). Supplemental data show time-lapse videos of this dynamic membrane blebbing under bright-field illumination (Supplemental Fig. 1) and with fluorescence imaging (Supplemental Fig. 2).

**Tipifarnib Evokes Plasmalemmal Ca\(^{2+}\) Influx but Not Ca\(^{2+}\) Release from Intracellular Stores.** Experiments were carried out to determine the source of Ca\(^{2+}\) mediating the tipifarnib effects. Figure 3A shows representative traces of [Ca\(^{2+}\)]\(_i\), recorded from cells in response to...
application of tipifarnib in the absence (control) and presence of preincubation (1 h) in ruthenium red (100 nM), an inhibitor of the mitochondrial uniporter. Preincubation in ruthenium red failed to inhibit the tipifarnib-induced Ca\textsuperscript{2+} elevation and resulted in increases in [Ca\textsuperscript{2+}], that were greater than those observed in control cells (tipifarnib alone). Likewise, depletion of intracellular Ca\textsuperscript{2+} stores with the sarcoplasmic/endoplasmic Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin did not inhibit the elevations in [Ca\textsuperscript{2+}], elicited by tipifarnib.

In identical experiments, preincubation in ruthenium red resulted in a statistically significant increase in the [Ca\textsuperscript{2+}], elevations evoked by tipifarnib, whereas thapsigargin had no effect on these changes in [Ca\textsuperscript{2+}]. Taken together, our data suggest that the mechanism by which tipifarnib increases [Ca\textsuperscript{2+}], in U937 cells does not involve liberation of Ca\textsuperscript{2+} from intracellular pools.

To determine whether tipifarnib elevates [Ca\textsuperscript{2+}], by promoting an influx of Ca\textsuperscript{2+} experiments were carried out whereby tipifarnib was applied in the absence and presence of extracellular Ca\textsuperscript{2+} (2.5 mM) and the pan-selective Ca\textsuperscript{2+} channel inhibitors La\textsuperscript{3+} (10 μM) and Gd\textsuperscript{3+} (100 μM). Figure 3C shows representative traces of [Ca\textsuperscript{2+}], recorded from U937 cells exposed to tipifarnib under the indicated conditions. U937 cells failed to exhibit increases in [Ca\textsuperscript{2+}], in response to tipifarnib when either extracellular calcium was removed from the bath solution or 100 nM La\textsuperscript{3+} was applied with the drug. In similar experiments, both depletion of extracellular Ca\textsuperscript{2+} or application of either La\textsuperscript{3+} or Gd\textsuperscript{3+} depressed the tipifarnib-induced elevations in [Ca\textsuperscript{2+}], in a statistically significant manner (Fig. 3D). 2-APB (100 μM), which blocks various plasma membrane Ca\textsuperscript{2+} channels, including store-operated Ca\textsuperscript{2+} channels, and the inositol trisphosphate receptor of the ER, did not prevent tipifarnib-induced increases in [Ca\textsuperscript{2+}]. On the contrary, when 2-APB was coapplied with tipifarnib the increases in [Ca\textsuperscript{2+}], were more pronounced than with tipifarnib alone (Fig. 3D). Membrane “boiling” was not observed in any of the conditions that prevented the elevations in [Ca\textsuperscript{2+}], (data not shown).

**Tipifarnib-Induced Ca\textsuperscript{2+} Influx Promotes Cell Death in Tumor Cells.** To establish a link between tipifarnib-induced influx of Ca\textsuperscript{2+} and tumor cell death, we tested the effects of the broad-spectrum Ca\textsuperscript{2+} channel inhibitor La\textsuperscript{3+} on the survival of U937 cells. Figure 4 shows the relative growth rate of U937 cells as a function of tipifarnib concentration in the absence and presence of 10 μM La\textsuperscript{3+}. Application of La\textsuperscript{3+} at a concentration shown to inhibit tipifarnib-induced increases in [Ca\textsuperscript{2+}], was associated with a statistically significant increase in the survival of U937 cells. Similar protection from tipifarnib-induced growth inhibition was observed with coapplication of Gd\textsuperscript{3+} (100 μM), but not with ruthenium red (100 nM) (data not shown). In contrast, 2-APB (100 μM), which potentiates tipifarnib-induced elevations in [Ca\textsuperscript{2+}], enhanced tipifarnib-induced cell death (Fig. 4). Similar effects were observed with lower concentrations of 2-APB (data not shown). Thus, our results imply that the increases in [Ca\textsuperscript{2+}], produced by tipifarnib contribute to the tumor cell death elicited by this drug.

**Tipifarnib Evokes Increases in [Ca\textsuperscript{2+}], in 8226 and 8226/R5 Cells.** To further explore the relationship between tipifarnib-induced cell death and increases in [Ca\textsuperscript{2+}], we tested the effects of tipifarnib in two additional cell lines that exhibit sensitivity and resistance to tipifarnib, 8226 and 8226/5R cells, respectively. Figure 5A shows representative traces of [Ca\textsuperscript{2+}], recorded from an 8226 cell and an 8226/R5 cell. Whereas 8226 cells exhibited robust elevations in [Ca\textsuperscript{2+}], noted for 8226/R cells were not as pronounced. Furthermore, 8226 cells, such as U937 cells, displayed apparent rapid decreases in [Ca\textsuperscript{2+}], that were associated with loss of fura-2 fluorescence, which is indicative of compromised plasma membrane integrity (Fig. 5A). In contrast, 8226/R5 cells displayed transient increases in [Ca\textsuperscript{2+}], after tipifarnib application, but the decrease in [Ca\textsuperscript{2+}], was not caused by a loss of fura-2 fluorescence (i.e., signal intensity at both 340- and 380-nM excitation was not diminished). The change in [Ca\textsuperscript{2+}], evoked by 5 μM tipifarnib in 8226 cells was >250% greater than that observed in 8226/R5 cells (Fig. 5B).

Experiments were carried out to determine whether 2-APB could also influence the response to tipifarnib in 8226 and 8226/R5 cells. Figure 5C shows traces of characteristic changes in [Ca\textsuperscript{2+}], observed in 8226 and 8226/R5 cells when tipifarnib (5 μM) was applied alone or with 100 μM 2-APB. The tipifarnib-induced increases in [Ca\textsuperscript{2+}], were potentiated by 2-APB in both cell lines. 2-APB amplified the tipifarnib-evoked increase in [Ca\textsuperscript{2+}], by ~400% in 8226 cells (Fig. 5D). In 8226/R5 cells the increase was ~250%, and this potentiation changed the amplitude of the response (226 ± 12 nM) such that it was comparable with that seen in 8226 cells in the absence of 2-APB (215 ± 17 nM) (Fig. 5D).

**U937 and 8226 Cells Express Higher Levels of Orai3 than 8226/R5 Cells.** One of the plasma membrane Ca\textsuperscript{2+} channels found in U937 cells is the channel mediating the
are unlikely to produce these effects, they raise the possibility that the Orai homolog, Orai3, may be involved. Unlike both Orai1 and Orai2, Orai3 channels are not inhibited by 2-APB (DeHaven et al., 2008), but rather potentiated by the compound even at high concentrations (100 μM) (DeHaven et al., 2008). This motivated us to retrospectively analyze gene array data we previously collected on the tipifarnib-sensitive cell lines U937 and 8226 and the tipifarnib-resistant 8226/R5 cells (Buzzeo et al., 2005; Hazlehurst et al., 2006). Figure 6A shows the results obtained and indicates a trend of Orai3 being expressed at lower levels in the 8226/R5 cells. No such trend was observed for the genes of other known proteins related to I_{CRAC} (i.e., Orai2 and STIM2) or other Ca^{2+} channels expressed in these cells (i.e., TRPM2) (data not shown).

To more precisely quantify the expression levels of Orai3 transcripts in U937, 8226 and 8226/R5 cells, real-time quantitative RT-PCR was carried out on mRNA extracts from the cancer cell lines. Figure 6B shows the mean RQ obtained for three experiments testing for Orai3 expression. Consistent with the gene array data, the levels of Orai3 measured in both U937 and 8226 cells were significantly greater than those measured in the tipifarnib-insensitive 8226/R5 cells.
Discussion

The major finding reported here is that tipifarnib activates a plasma membrane channel in U937 and 8226 cells that produces pronounced elevations in \([\text{Ca}^{2+}]_{\text{i}}\), that consequently evoke loss of membrane integrity. The increases in \([\text{Ca}^{2+}]_{\text{i}}\), elicited by tipifarnib result in ER stress, and the drug-induced cell death may be diminished or enhanced by blocking or potentiating these changes in \([\text{Ca}^{2+}]_{\text{i}}\), respectively. The pharmacological characteristics of the channel activated by tipifarnib are consistent with Orai3-containing store-operated channels. Moreover, mRNA transcripts for this SOC subunit are expressed at higher levels in tumor cell lines that have greater sensitivity to tipifarnib.

Previously, we have shown that both tipifarnib and bortezomib as single agents and in combination overcome cell adhesion-mediated drug resistance and induce ER stress in multiple myeloma and acute myeloid leukemia cells (Buzzeo et al., 2005; Yanamandra et al., 2006). Our data show that that tipifarnib, as a single agent, processed caspase-12, an ER resident caspase in a dose-dependent manner (Fig. 1A). Bortezomib has been shown to induce ER stress and dysregulate \([\text{Ca}^{2+}]_{\text{i}}\), in multiple myeloma cells by promoting mito-

Fig. 5. The tipifarnib-resistant cell line 8226/R5 shows depressed \([\text{Ca}^{2+}]_{\text{i}}\), responses to tipifarnib that are potentiated by 2-APB. A, representative traces of \([\text{Ca}^{2+}]_{\text{i}}\), recorded from an 8226 cell (black trace) and an 8226/R5 cell (gray trace) exposed to 5 \(\mu\)M tipifarnib. The black arrow indicates the start of tipifarnib (5 \(\mu\)M) application. Tipifarnib was continuously applied for the remainder of the recording. B, mean peak change in \([\text{Ca}^{2+}]_{\text{i}}\), produced by the application of tipifarnib (5 \(\mu\)M) in 8226 (n = 177) and 8226/R5 (n = 243) cells. * indicates significant difference (p < 0.001). C, representative traces of \([\text{Ca}^{2+}]_{\text{i}}\), recorded from four cells (8226 cells, black traces; 8226/R5 cells, gray traces) exposed to 5 \(\mu\)M tipifarnib in the absence (solid traces) and presence (dashed traces) of 100 \(\mu\)M 2-APB. The black arrow indicates the start of tipifarnib (5 \(\mu\)M) application. Tipifarnib was continuously applied for the remainder of the recording. 2-APB was applied for 5 min before and during tipifarnib application. D, mean peak change in \([\text{Ca}^{2+}]_{\text{i}}\), observed in 8226 and 8226/R5 cells upon application of 5 \(\mu\)M tipifarnib alone (8226; n = 63) and 8226/R5 (n = 47) or tipifarnib in combination with 100 \(\mu\)M 2-APB (8226, n = 45; 8226/R5, n = 82). * indicate significant difference from tipifarnib alone for each cell type (p < 0.001), and # denote significant difference between the cell types within each treatment group (i.e., tipifarnib alone or tipifarnib + 2-APB).

Fig. 6. Higher expression of Orai3 mRNA in tipifarnib-sensitive leukemia cell lines. A, bar graph of Orai3 gene expression levels detected in U937, 8226, and 8226/R5 using an Affymetrix U133A human gene array. Signal represents the raw expression value recorded with the gene chip. Bars for 8226 and 8226/R5 represent mean of data collected from two separate chips. B, mean RQ (±S.E.M.) of Orai3 transcripts detected in mRNA extracts from U937, 8226, and 8226/R5 cells. * indicates significant difference from 8226 cells, and # denotes significant difference in Orai3 expression between U937 and 8226/R5 cells (n = 3 and p < 0.05 for all).
chondrial Ca\(^{2+}\) release (Landowski et al., 2005). The findings presented here show that tipifarnib dysregulates [Ca\(^{2+}\)], in U937 and 8226 cells via a mechanism distinct from that of bortezomib, because inhibition of the Ca\(^{2+}\) uniporter of the mitochondria failed to inhibit the tipifarnib effect.

FTI-277 and FTI-2153 did not stimulate elevations in [Ca\(^{2+}\)], at concentrations previously shown to have cellular effects (Lerner et al., 1995; Sun et al., 1999) (Fig. 2B), suggesting that inhibition of farnesyltransferase alone is not sufficient to elicit the [Ca\(^{2+}\)] overload. It is noteworthy that the FTI perillyl alcohol has also been shown to elevate Ca\(^{2+}\), induce membrane boiling, and promote apoptosis in activated T lymphocytes (Wei et al., 2000). The effects of perillyl alcohol on T lymphocyte, however, were biphasic and more rapid than those reported here for tipifarnib (<1 min). The precise mechanism by which perillyl alcohol elevates [Ca\(^{2+}\)] has not been identified, and it is unknown if this compound elevates [Ca\(^{2+}\)], in tumor cells.

Several studies have linked apoptosis to calcium signaling pathways as a response to cellular stress (Orrenius et al., 2003). ER stress induces apoptosis via two different mechanisms, the unfolded protein response and dysregulation of Ca\(^{2+}\) homeostasis (Ferri and Kroemer, 2001; Lee et al., 2002). Our findings that agents that inhibit or potentiate tipifarnib-induced [Ca\(^{2+}\)] overload diminish or enhance the apoptosis promoted by this drug, respectively, indicate that this influx of Ca\(^{2+}\) through the plasma membrane is directly linked to the influence of tipifarnib on cell survival. Moreover, the plasma membrane “boiling” observed here is known to be triggered by [Ca\(^{2+}\)] overload and is a marker for apoptotic cell death (Laster et al., 1988; Furuya et al., 1994). Such membrane blebbing was not observed in the absence of extracellular Ca\(^{2+}\) or when lanthanides were applied.

Several plasma membrane calcium channels have been identified in U937 cells that may potentially account for the elevations in [Ca\(^{2+}\)], and concomitant cell death reported here in response to tipifarnib application. They include TRPM2, TRPM7, and store-operated calcium channels (Wilmott et al., 1996; Lee et al., 2006; Zhang et al., 2006). In U937 cells, activation of TRPM2 with either H\(_2\)O\(_2\) or tumor necrosis factor-\(\alpha\) increased cleavage of caspase-8, caspase-9, caspase-3, caspase-7, and PARP, and, consequently, cell death was enhanced in a [Ca\(^{2+}\)]-dependent manner (Zhang et al., 2006). However, TRPM2 channels are not inhibited by concentrations of La\(^{3+}\) \(\leq\) 100 \(\mu\)M (Kraft et al., 2004), and low concentrations of this trivalent cation blocked both the Ca\(^{2+}\) increases and cell death evoked by tipifarnib in our study. Furthermore, another study suggests that TRPM2 channels heterologously expressed in HEK-293 cells are blocked by 2-APB with an IC\(_{50}\) of 1 \(\mu\)M (Togashi et al., 2008). Thus, the pharmacological properties of the tipifarnib-activated conductance are not consistent with TRPM2.

Like TRPM2, TRPM7 is activated by H\(_2\)O\(_2\) and is believed to be involved in oxidative stress-induced cell death. Overexpression of TRPM7 in HEK-293 cells results in cell swelling, detachment, and death (Nadler et al., 2001). In cortical neurons, small interfering RNA-mediated depression of TRPM7 expression protected cells from anoxic cell death (Aarts and Tymianski, 2005). However, 10 \(\mu\)M La\(^{3+}\) and Gd\(^{3+}\) failed to inhibit TRPM7 channels heterologously expressed in HEK-293 cells (Monteilh-Zoller et al., 2003), and 1 mM concentrations of these ions were required to inhibit TRPM7. Thus, the tipifarnib-activated Ca\(^{2+}\) conductance does not share these properties with TRPM7, because 200 nM La\(^{3+}\) was sufficient to abolish both the Ca\(^{2+}\) elevations and the cell death evoked by the FTI, and 100 \(\mu\)M Gd\(^{3+}\) produced a near-complete block of the Ca\(^{2+}\) increase. In addition,100 \(\mu\)M 2-APB blocks \(~25\%\) of TRPM7-evoked currents (Li et al., 2006), but fails to decrease the Ca\(^{2+}\) elevations or U937 cell death evoked by tipifarnib.

To date, there is little evidence linking the activation of store-operated calcium channels and cell death. A study on cervical epithelial cells indicates that soft substrate-induced apoptosis is mediated by the interaction of the calcium sensor, stromal interacting molecule 1, and the pore-forming subunit (Orai1) of store-operated calcium channels (Chiu et al., 2008). Upon contact with soft substrate, SOC activity is up-regulated and Ca\(^{2+}\) homeostasis is disturbed in normal cervical epithelial cells, which in turn triggers a proapoptotic signaling cascade involving \(\mu\)-calpain (Chiu et al., 2008). It is noteworthy that the activation of SOC is depressed in malignant cervical epithelial cells, and these cells do not undergo soft substrate-induced apoptosis (Chiu et al., 2008). The inhibition of tipifarnib-induced Ca\(^{2+}\) influx by La\(^{3+}\) and Gd\(^{3+}\) is consistent with an Orai1-containing SOC-mediated phenomenon. However, Orai1-containing SOC are inhibited by 100 \(\mu\)M 2-APB, thus the activation the potentiation of tipifarnib effects by this drug is inconsistent with an Orai1-mediated effect. Unlike Orai1-containing channels, the related SOC subunit, Orai3, has been shown to form functional SOC that are potentiated by 2-APB (DeHaven et al., 2008). Gene array data and quantitative RT-PCR experiments conducted here confirm that Orai3 is expressed in both U937 and 8226 cells. It is noteworthy that the 8226/R5 daughter cell line, which is resistant to tipifarnib, expresses Orai3 at significantly lower levels than the tipifarnib-sensitive 8226 parent line. Coapplication of 2-APB with tipifarnib potentiates the effects of tipifarnib on the resistant 8226/R5 cells, indicating that greater tipifarnib sensitivity may be conferred in this manner. It is noteworthy that a study on breast cancer cell lines found that Orai3-containing channels are expressed at higher levels in estrogen receptor-positive cells relative to estrogen receptor-negative tumor cells (Motiani et al., 2010). These channels were also functionally identified on the basis of 2-APB sensitivity, with Orai3-expressing cells having SOC responses potentiated by the drug. However, the capacity of these Orai3 channels to influence cell survival was not addressed in that study, nor was the sensitivity of these channels to chemotherapeutic agents assessed.

In conclusion, in this study, we identify a novel mechanism by which tipifarnib induces ER stress in U937 leukemia cells. Tipifarnib activates a plasma membrane Ca\(^{2+}\) channel with pharmacological properties consistent with Orai3 in both U937 and 8226 cells. The long-lived opening of this channel by tipifarnib results in cytoplasmic Ca\(^{2+}\) overload, membrane boiling, and loss of membrane integrity. Pharmacological manipulations that depress or enhance these effects of tipifarnib on [Ca\(^{2+}\)], elicit concomitant changes in tumor cell survival. Our data also suggest that designing and developing novel chemotherapy agents that specifically target the ER stress-related pathway may have beneficial effect to overcome de novo drug resistance in patients with multiple myeloma and acute myeloid leukemia.
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**Authorship Contributions**

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**References**


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