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ZAK is required for doxorubicin, a novel ribotoxic stressor, to induce SAPK activation and apoptosis in HaCaT cells

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Doxorubicin is an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors. The stress-activated protein kinases (SAPKs) are frequently activated by a number of cancer chemotherapeutics. When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Some inhibitors of protein synthesis, known as ribotoxic stressors, coordinately activate SAPKs and lead to apoptotic cell death. We demonstrate that doxorubicin effectively inhibits protein synthesis, activates SAPKs, and causes apoptosis. Ribotoxic stressors share a common mechanism in that they require ZAK, an upstream MAP3K, to activate the pro-apoptotic and proinflammatory signaling pathways that lie downstream of SAPKs. By employing siRNA mediated knockdown of ZAK or administration of sorafenib and nilotinib, kinase inhibitors that have a high affinity for ZAK, we provide evidence that ZAK is required for doxorubicin-induced proinflammatory and apoptotic responses in HaCaT cells, a pseudo-normal keratinocyte cell line, but not in HeLa cells, a cancerous cell line. ZAK has two different isoforms, ZAK-α (91 kDa) and ZAK-β (51 kDa). HaCaT or HeLa cells treated with doxorubicin and immunoblotted for ZAK displayed a progressive decrease in the ZAK-α band and the appearance of ZAK-β bands of larger size. Abrogation of these changes after exposure of cells to sorafenib and nilotinib suggests that these alterations occur following stimulation of ZAK. We suggest that ZAK inhibitors such as sorafenib or nilotinib may be effective when combined with doxorubicin to treat cancer patients.

Introduction

Doxorubicin (dox, adriamycin) is an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors.[1] Several mechanisms for the chemotherapeutic actions of doxorubicin have been proposed, including: (a) intercalation into DNA, leading to inhibition of macromolecular synthesis; (b) generation of reactive oxygen species (ROS), leading to DNA damage or lipid peroxidation; and (c) inhibition of topoisomerase II, followed by DNA damage. Doxorubicin-mediated apoptotic cell death is likely a response to one or more of these upstream actions.[1-3] The clinical efficacy of doxorubicin is limited by both acute and chronic complications. Patients receiving doxorubicin frequently present with acute side effects such as fatigue, nausea/vomiting, pain, sleep disturbances, cachexia and depression.[4] In addition, patients may develop cardiomyopathy, leading to life-threatening congestive heart failure. Cardiomyopathy frequently correlates with the total amount of administered drug.[3] Production of oxygen radicals has been proposed for doxorubicin-mediated cardiotoxicity, whereas the inhibition of both topoisomerase enzyme

and DNA synthesis is thought to underlie doxorubicin-induced death of tumor cells.[3,5] Identifying the mechanism(s) by which normal and healthy cells respond differentially to doxorubicin may present opportunities to decrease the toxicity of doxorubicin on normal tissues while maintaining the efficacy of doxorubicin as an anti-cancer drug.

The stress-activated protein kinases (SAPKs), p38 mitogen-activated protein kinase (p38 MAPK) and Jun N-terminal kinase (JNK), are frequently activated by a number of cancer chemotherapeutics.[4] When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Doxorubicin is known to induce the activation of SAPKs in a number of normal cell types, including hepatocytes,[6] primary mouse macrophages[7] and cardiomyocytes.[8,9] Inhibitors of p38 MAPK and JNK have been employed to determine whether activation of SAPKs contributes either to the efficacy or to the undesirable side effects of doxorubicin. Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin, a related anthracycline.[8,9] Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but

do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line.[7]

The intercalation of doxorubicin into DNA is thought to comprise a major mode of action in producing cell death. In addition to its effects on DNA, doxorubicin also causes RNA damage[10] and inhibits the synthesis of DNA, RNA and proteins.[11-14] Some inhibitors of protein synthesis, known as ribotoxic stressors, activate SAPKs.[15] Well-known ribotoxic stressors include anisomycin, blasticidin, ricin, Shiga toxin, sarcin and ultra-violet radiation.[15,16] Here we demonstrate that doxorubicin effectively inhibits protein synthesis, activates JNK and p38 MAPK, and causes apoptosis.

Ribotoxic stressors share a common mechanism in that they require ZAK, an upstream MAP3K, to activate the pro-apoptotic and proinflammatory signaling pathways that lie downstream of p38 MAPK and JNK.[17,18] SiRNA-mediated knockdown of ZAK or administration of a small molecule inhibitor (DHP-2) of ZAK, suppress anisomycin- and UV-induced apoptosis and the phosphorylation of p38 MAPK and JNK.[17] Inhibition of ZAK also suppresses Shiga toxin- and ricin-induced SAPK activation and improves cell viability.[18] By employing siRNA mediated knockdown of ZAK or administration of sorafenib and nilotinib, kinase inhibitors that have a high affinity for ZAK, we provide evidence that ZAK is required for doxorubicin-induced proinflammatory and apoptotic responses in HaCaT cells, a pseudo-normal human keratinocyte cell line, but not in HeLa cells.

Results

Doxorubicin-induced MAPK activation and apoptosis are decreased after siRNA-mediated knockdown of ZAK. Ribotoxic stressors are characterized by their ability to activate JNK and p38 MAPK through inhibition of protein synthesis.[15] In many cell types, ZAK, a MAP3K, is required for ribotoxic stressors to be capable of activating the MAPKs, through which these kinases modulate the transcription of downstream genes.[17,18] In order to determine whether doxorubicin activates JNK and p38 MAPK, we exposed HaCaT cells to varying concentrations of doxorubicin and detected the increased phosphorylation of JNK and p38 MAPK by immunoblotting against an antibody specific for the phosphorylated form of JNK and p38 MAPK. Phosphorylation of these MAPKs was detected 24 h after addition of doxorubicin (5 to 50 µM; **Fig. 1A**). To determine whether ZAK is required for doxorubicin to induce the phosphorylation of JNK and p38 MAPK, we employed siRNA directed against the 5' portion of ZAK mRNA. SiRNA-mediated knockdown of ZAK strongly suppressed the phosphorylation of JNK and p38 MAPK, determined in cells that were harvested 24 h after the addition of doxorubicin (**Fig. 1A**). Interestingly, the knockdown of ZAK decreased the basal phosphorylation of p38 MAPK (**Fig. 1A**).

Similar to other cancer chemotherapeutic agents, doxorubicin induces apoptosis both in vivo[19-22] and in vitro.[2,8,9] To determine whether the pro-apoptotic effects of doxorubicin are regulated through activation of ZAK, we examined the cleavage of poly (ADP-ribose) polymerase (PARP) (from 116 kDa to 89 kDa) and caspase-3 (from procaspase to p20/p19 and p17 subunits), which are frequently employed as indicators of apoptosis. Knockdown

of ZAK suppressed the doxorubicin-induced cleavage of both PARP and caspase-3 in HaCaT cells (**Fig. 1A**).

To investigate the effects of knockdown of ZAK on doxorubicin-mediated cell death by morphological criteria, we analyzed HaCaT cells after staining with a nuclear fluorescent dye, bisbenzimide (**Fig. 1B**). Cells transfected with either scrambled (**Fig. 1B, i**) or ZAK-specific (**Fig. 1B, ii**) siRNA, but not treated with doxorubicin, demonstrated similar morphological features with the majority of the cells displaying uniform nuclear staining. A minority population in both groups displayed spontaneous apoptosis, manifested by brightly-fluorescent pyknotic or karyorrhetic nuclei (**Fig. 1B, i and ii**, arrowheads). We attribute these basal levels of apoptosis to the 24 hrs serum-deprivation. HaCaT cells transfected with scrambled siRNA and treated with doxorubicin (25 µM for 24 hrs) displayed extensive post-apoptotic secondary necrosis, characterized by massive loss of normal cellular and nuclear morphology, decreased cellular chromatin content and the appearance of ring-like chromatin aggregates adjacent to the plasma membrane/cell borders (**Fig. 1B, iii**, arrows). Extracellular release of chromatin was evident in some areas as localized, pericellular increase of background fluorescence (not shown). In contrast, cells transfected with ZAK-specific siRNA and treated with doxorubicin (25 µM for 24 hrs) displayed markedly preserved normal cellular and nuclear morphologies (**Fig. 1B, iv**).

To ensure that the ZAK siRNA did not produce off-target effects, we employed an additional sequence of siRNA directed against the 5' portion of ZAK mRNA (**Fig. 1C**). SiRNA-mediated knockdown of ZAK using sequence #2 also suppressed the doxorubicin-induced phosphorylation of JNK and p38 MAPK. Additionally, siRNA-mediated knockdown of ZAK using sequence #2 suppressed the doxorubicin-induced cleavage of PARP, although not as effectively as sequence #1. For this reason, we employed sequence #1 in subsequent experiments.

Doxorubicin-induced inhibition of protein translation measured by incorporation of [³H]-leucine. An invariant feature of ribotoxic stressors is their ability to inhibit protein translation.[15] To determine if doxorubicin inhibits protein synthesis, we exposed HaCaT cells to doxorubicin (1 to 25 µM) for varying times (6, 12 or 24 h), at which times cells were exposed to [³H]-leucine for 30 min. Exposure to doxorubicin at concentrations of 2.5 µM or greater resulted in a progressive decrease in the incorporation of [³H]-leucine (**Fig. 1B**). Cells treated with 2.5 µM doxorubicin decreased incorporation of [³H]-leucine to approximately 35% by the end of 24 h; treatment with 10 and 25 µM reduced levels of [³H]-leucine incorporation to below 10% at 24 h. Continuous examination of cells by microscopy demonstrated insignificant cell detachment, even 24 h after addition of doxorubicin.

Emetine blocks MAPK activation after a high dose of doxorubicin. Transduction by ribotoxic stressors of signals that lead to activation of SAPKs requires that the ribosomes be involved in protein synthesis at the time that cells are exposed to the stressor.[15] Blockade of protein synthesis by fast-acting inhibitors such as emetine, prior to the exposure of cells to ribotoxic stressors, prevents transduction of the signal(s) that lead to activation of JNK and p38 MAPK. Iordanov, et al. demonstrated
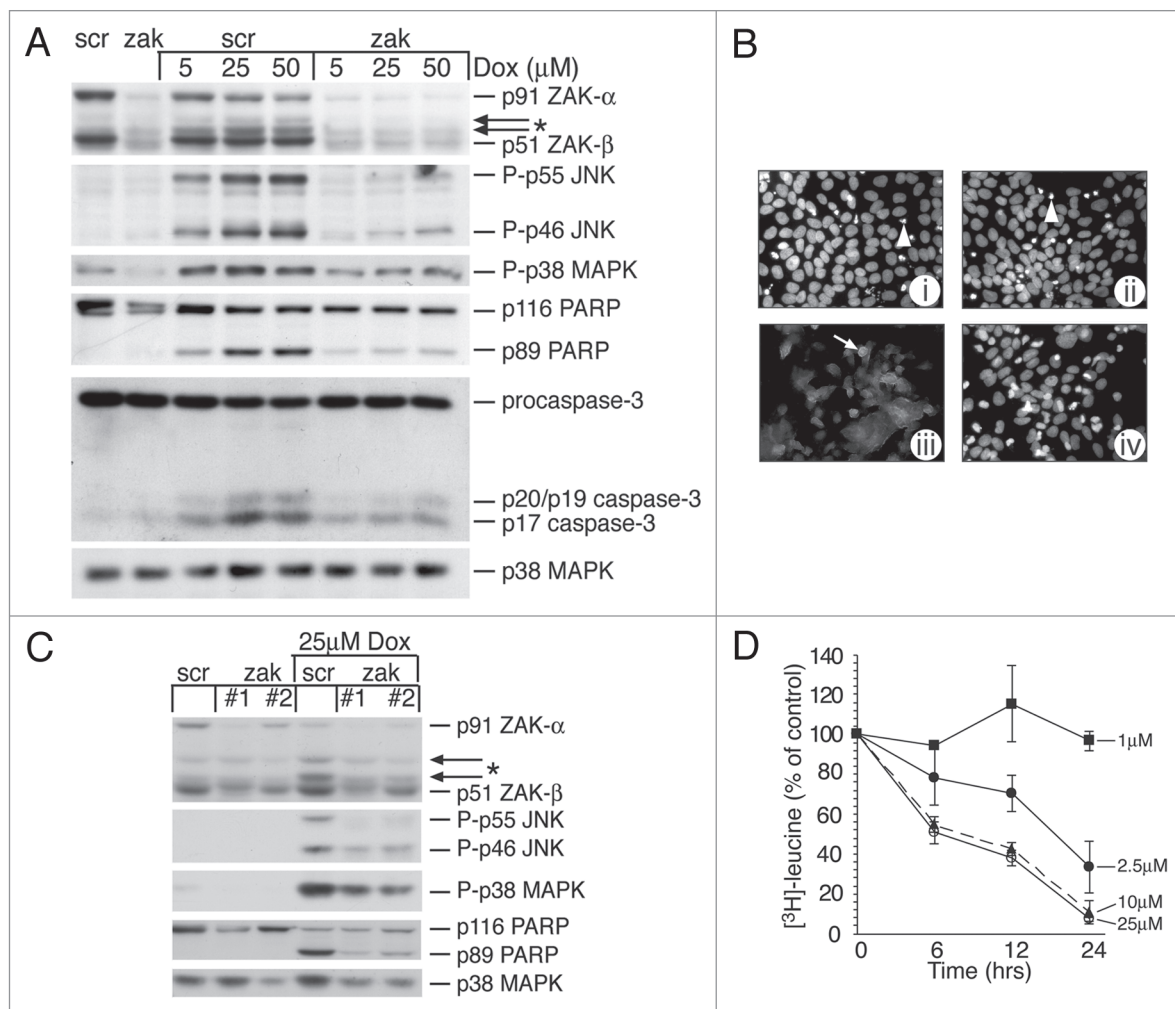
Figure 1. Effect of ZAK knockdown on doxorubicin-induced apoptosis, activation of SAPKs, and inhibition of protein synthesis. (A) HaCaT cells were exposed to ZAK-specific siRNA (zak) or scrambled siRNA (scr) prior to treatment for 24 h with vehicle or doxorubicin (5, 25 or 50 μM), as described in *Materials and Methods*. Lysates were collected and subjected to SDS-PAGE followed by western blot analysis with the antibodies indicated. (B) HaCaT cells grown on glass coverslips were exposed to scrambled siRNA (i and iii) or ZAK-specific siRNA (ii and iv) prior to treatment for 24 h with vehicle (i and ii) or 25 μM doxorubicin (iii and iv). Cells were exposed to bisbenzimide to examine fluorescent nuclear staining as described in Materials and Methods. (C) HaCaT cells were exposed to scrambled siRNA (scr) or ZAK-specific siRNA (zak) sequence #1 from (A) or sequence #2 prior to treatment for 24 h with vehicle or 25 μM doxorubicin, as described in Materials and Methods. Lysates were collected and subjected to SDS-PAGE followed by western blot analysis with the antibodies indicated. (D) HaCaT cells were treated in triplicate for 6, 12 or 24 hours with either vehicle or 1, 2.5, 10 or 25 μM of doxorubicin. Cells were pulse-labeled with [³H]-leucine for the final 30 minutes of treatment.

that emetine blocked protein synthesis in less than 1 minute after the addition to cells.[15] To determine whether prior treatment of HaCaT cells with emetine would block the activation of JNK and p38 MAPK, cells were exposed to emetine or vehicle prior to the addition of doxorubicin. We employed a high concentration of doxorubicin (0.5 mM) to induce the rapid phosphorylation of JNK and p38 MAPK. Doxorubicin induced the phosphorylation of JNK and p38 MAPK at 2 h, but not at 1 h or earlier (**Fig. 2A**). Addition of emetine prior to the exposure to doxorubicin completely blocked the phosphorylation of JNK and p38 MAPK (**Fig. 2A**). Doxorubicin suppressed the incorporation of [³H]-leucine by 50% at 1 h and completely at 2 h. We performed a similar experiment using $CdCl_2$, which is not a ribotoxic stressor[23] and leads to the activation of JNK and p38 MAPK through other mechanisms. In contrast to doxorubicin, the phosphorylation of JNK and p38 MAPK was not suppressed by emetine (**Fig. 2C**).

Inhibitors of ZAK block doxorubicin-induced apoptosis and MAPK activation in HaCaT cells. An important goal in cancer chemotherapy is to reduce collateral damage in normal tissues and organs. The administration of effective doses of doxorubicin to cancer patients is frequently limited by the potential for development of cardiotoxicity and other adverse responses.[3] Identification of agents that could selectively suppress the destruction of normal tissue by doxorubicin may permit the administration of larger or more frequent doses of doxorubicin to cancer patients. Previous studies have demonstrated that inhibition of ZAK by an experimental small molecule inhibitor (DHP-2; Eli Lilly) reduces ribotoxic stressor-induced cell death.[17,18] However,
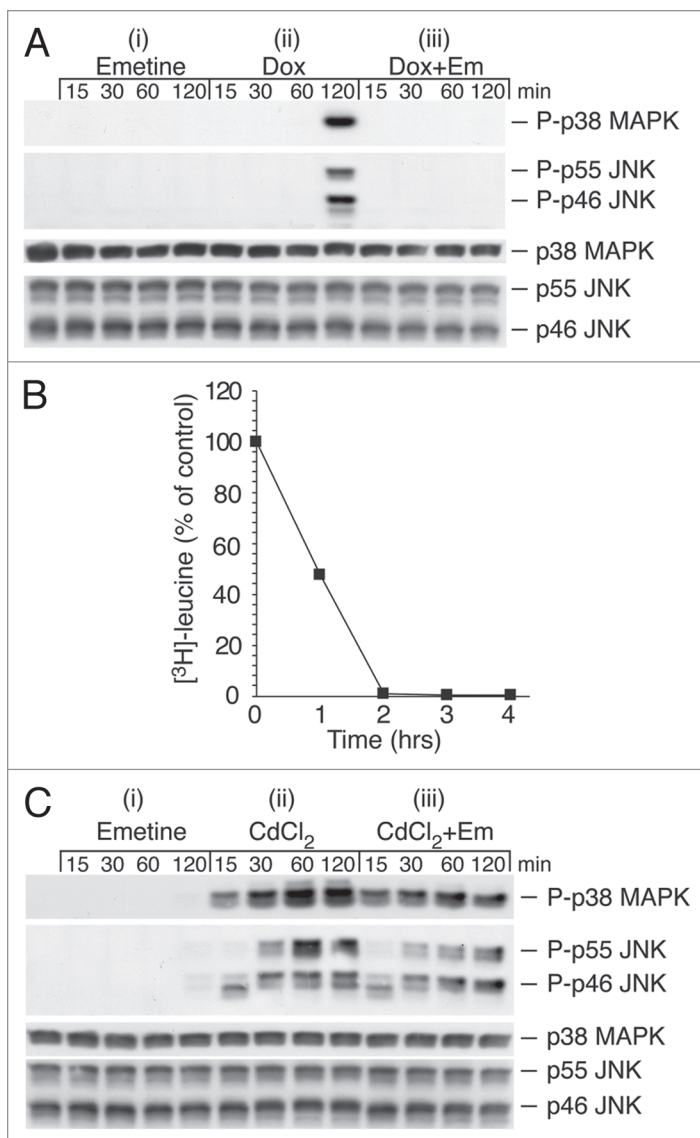
Figure 2. Inhibition of doxorubicin-induced activation of SAPKs by pretreatment with emetine. (A) HaCaT cells were pretreated with vehicle (ii) or emetine (100 µg/mL; i and iii) for 30 minutes prior to stimulation with doxorubicin (0.5 mM; ii and iii) for 15, 30, 60 or 120 min. Lysates were collected and submitted to SDS-PAGE, followed by western blot analysis with the antibodies as indicated. (B) HaCaT cells were treated in triplicate for 1, 2, 3 or 4 hours with vehicle or 0.5 mM doxorubicin. Cells were pulse-labeled with [³H]-leucine for the final 30 minutes of treatment. (C) HaCaT cells were pretreated with vehicle (ii) or emetine (100 µg/mL; i and iii) for 30 minutes prior to stimulation as indicated with CdCl₂ (100 µM; ii and iii) for 15, 30, 60 or 120 min. Lysates were collected and subjected to SDS-PAGE, followed by western blot analysis with the antibodies as indicated.

DHP-2 is no longer produced by Eli Lilly and is unavailable. In a comprehensive effort to identify the target of 38 small molecule kinase inhibitors, Karaman et al. determined the dissociation constants of a panel of 287 distinct protein kinases, including ZAK.[24] Sorafenib (Bayer), a multi-kinase inhibitor that has been employed in the treatment of renal cell carcinoma and hepatocellular carcinoma, was found to have a very high binding affinity for ZAK (K_d = 6.3 nM).[24] In one trial for hepatocellular

carcinoma, patients who received sorafenib and doxorubicin together had significantly longer median durations of overall survival and progression-free survival than patients receiving doxorubicin alone.[25] Another small molecule kinase inhibitor with a high binding affinity for ZAK is nilotinib (Novartis, K_d = 3 nM), which also inhibits breakpoint cluster region-abelson (BCR-ABL) and is currently in clinical use for treatment of chronic myelogenous leukemia.[26] Although the binding affinities of sorafenib and nilotinib for ZAK have been reported, neither agent has been tested for their ability to inhibit ZAK activity.

To determine whether sorafenib or nilotinib would inhibit downstream actions of ZAK, we administered these agents (1 µM) to HaCaT cells 30 min prior to treatment with doxorubicin (10 µM or 25 µM) for 24 h. The presence of either inhibitor strongly suppressed doxorubicin-induced phosphorylation of JNK and p38 MAPK (**Fig. 3A**). Just as in HaCaT cells exposed to ZAK siRNA, exposure of these cells to sorafenib or nilotinib decreased the basal phosphorylation of p38 MAPK (**Fig. 3A**). Sorafenib and nilotinib also reduced the cleavage of PARP and caspase-3, suggesting that doxorubicin-mediated apoptosis was also suppressed.

ZAK inhibitors block daunorubicin-induced apoptosis and MAPK activation in HaCaT cells. Daunorubicin is an anthracycline that is considered to act by similar mechanisms as doxorubicin but shows less potent antitumor activity.[3] To determine whether the inhibition of ZAK effects daunorubicin-induced apoptosis and MAPK activation, we pretreated HaCaT cells with sorafenib or nilotinib (1 µM) followed by daunorubicin (2.5, 10, 25 µM) for 24 h. Similar to the experiments with doxorubicin, the presence of either inhibitor strongly suppressed daunorubicin-induced phosphorylation of JNK and p38 MAPK (**Fig. 3B**). Sorafenib and nilotinib also reduced the cleavage of PARP and caspase-3, suggesting that daunorubicin-mediated apoptosis was also suppressed.

Inhibitors of JNK or p38 partially block doxorubicin-induced apoptosis in HaCaT cells. ZAK is a MAP3K that has been shown to induce the phosphorylation of p38 MAPK and JNK. To determine whether suppression of JNK or p38 MAPK would inhibit doxorubicin-induced apoptosis, we administered SB 203580 (10 µM), SP 600125 (20 µM), or both in combination to HaCaT cells 30 min prior to treatment with 25 µM doxorubicin for 24 h. The presence of either inhibitor or a combination of both resulted in diminished cleavage of PARP and caspase-3, suggesting that JNK and p38-MAPK participated to an extent in doxorubicin-mediated apoptosis. In the presence of a pancaspase inhibitor, zVAD-fmk, doxorubicin-induced apoptosis was completely inhibited (**Fig. 3C**).

ZAK inhibitors and ZAK siRNA do not block doxorubicin-induced apoptosis in HeLa cells. To test whether ZAK inhibitors would reduce cell death in a cancerous cell line we pretreated HeLa cells with sorafenib or nilotinib (1 µM) followed by doxorubicin (10 µM or 25 µM) for 24 h. In contrast to their ability to suppress PARP and caspase-3 cleavage in HaCaT cells, sorafenib and nilotinib failed to reduce PARP or caspase-3 cleavage in HeLa cells (**Fig. 4A**). In HeLa cells, doxorubicin failed to increase the

phosphorylation of JNK and p38 MAPK, perhaps because the basal levels of these phosphorylated SAPKs were already elevated in the absence of an inducer. Nevertheless, the phosphorylation of SAPKs was suppressed by sorafenib and nilotinib, suggesting that the inhibitors were capable of suppressing ZAK in these cells. These data suggest that the elevated endogenous activity of ZAK in HeLa cells may be responsible for the increased basal phosphorylation of JNK and p38 MAPK.

To test whether ZAK siRNA would reduce doxorubicin-mediated apoptosis in HeLa cells, we employed ZAK-targeting siRNA. SiRNA-mediated knockdown of ZAK slightly reduced doxorubicin-mediated cleavage of PARP and caspase-3 in HeLa cells (**Fig. 4B**), indicating that the pro-apoptotic actions of doxorubicin in these cells was mediated in part through activation of ZAK.

Doxorubicin-induced alterations of ZAK protein. ZAK has two different isoforms, ZAK-α and ZAK-β. ZAK-α has an apparent molecular weight of 91 kDa. ZAK-β is a shorter species of ZAK (51 kDa) because it lacks several exons in the coding region and, compared to ZAK-α, has a distinct C-terminus.[18] When HaCaT or HeLa cells were treated with doxorubicin and immunoblotted for ZAK, we noticed that the ZAK-α band decreased in intensity (**Figs. 1A and 4B**). In addition, bands of slightly higher molecular weight appeared above the 51 kDa ZAK-β band (**Figs. 1A* and 4B***).

To determine the kinetics of the disappearance of the ZAK-α band and the appearance of slightly higher molecular weight bands above ZAK-β, we added 25 µM of doxorubicin to HaCaT cells and harvested at 4-hour intervals up to 24 hours for immunoblotting with ZAK Ab. The higher molecular weight bands above ZAK-β appeared 8 hours after doxorubicin treatment and increased in intensity thereafter. The disappearance of the 91 kDa ZAK-α began 16 hours after doxorubicin treatment (**Fig. 5A**).

To determine if the doxorubicin-induced disappearance of the ZAK-α band and the appearance of the higher molecular weight bands above ZAK-β were due to phosphorylation, we exposed lysates to calf intestinal phosphatase (CIP). The presence of CIP did not alter the disappearance or appearance of the ZAK bands, indicating that neither was a result of phosphorylation (data not shown). Immunoblotting with phospho-p38 confirmed the efficacy of the phosphatase treatment (data not shown).

To determine if the doxorubicin-induced changes in the two ZAK isoforms could result from ubiquitin-mediated proteolysis, we utilized MG-132, an inhibitor of proteasomal degradation. The presence of the MG-132 compound did not affect the disappearance of the 91 kDa ZAK-α band, suggesting that its disappearance was not proteasome dependent (**Fig. 5B**). By contrast, the higher molecular weigh bands above ZAK-β increased in intensity in the presence of the MG-132 compound, suggesting that these bands undergo proteasome-mediated degradation after doxorubicin treatment.
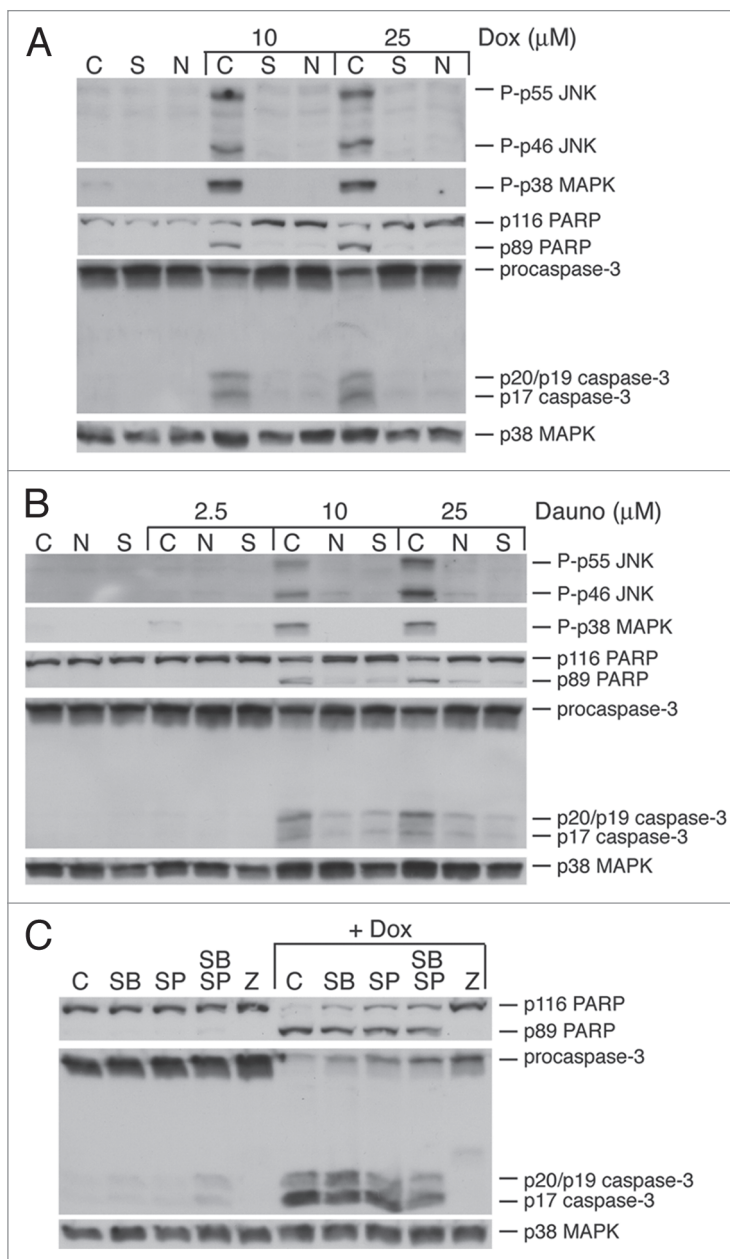

Figure 3. Effect of inhibitors on doxorubicin- and daunorubicin-induced apoptosis and activation of SAPKs. HaCaT cells were pretreated for 30 minutes with vehicle or inhibitors. Twelve hours later an additional dose of inhibitor was added to each well. Twenty-four hours after the addition of doxorubicin or daunorubicin, lysates were collected and subjected to SDS-PAGE followed by western blot analysis with the antibodies as indicated. (A) HaCaT cells were pretreated with vehicle (C), sorafenib (S; 1 µM), or nilotinib (N; 1 µM) for 30 minutes, followed by treatment with doxorubicin (10 or 25 µM) for 24 h. (B) HaCaT cells were pretreated with vehicle (C), sorafenib (S; 1 µM), or nilotinib (N; 1 µM) for 30 minutes, followed by treatment with daunorubicin (2.5, 10 or 25 µM) for 24 h. (C) HaCaT cells were pretreated with vehicle (C), SB 203580 (SB; 10 µM), SP 600125 (SP; 20 µM), both (SB SP), or zVAD-fmk (Z; 25 µM) for 30 minutes, followed by treatment with doxorubicin (25 µM) for 24 h.
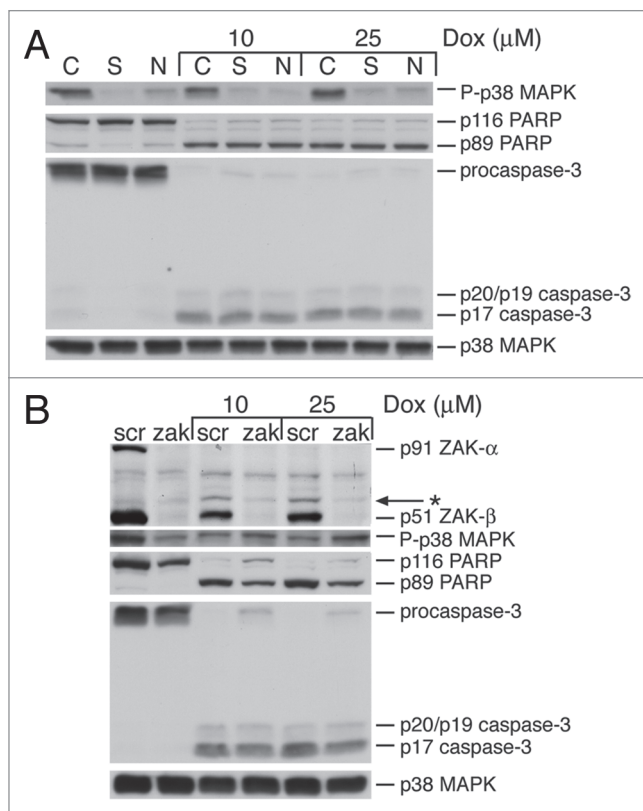
Figure 4. Effect of ZAK knockdown, sorafenib or nilotinib on doxorubicin-induced apoptosis in HeLa cells. Following exposure to 10 or 25 μM doxorubicin, HeLa cell lysates were collected and subjected to SDS-PAGE, followed by western blot analysis with the antibodies as indicated. (A) Cells were pretreated with vehicle (C), sorafenib (S; 1 μM), or nilotinib (N; 1 μM) for 30 minutes, followed by treatment with doxorubicin for 24 h. At 12 h an additional dose of vehicle or inhibitors was added to each well. (B) Cells were exposed to scrambled siRNA (scr) or ZAK-specific siRNA (zak) as described in Materials and Methods, followed by treatment for 24 h with vehicle or doxorubicin.

Figure 5. Effect of inhibition of ZAK, p38 MAPK or JNK on doxorubicin-induced degradation or modification of ZAK. (A) HaCaT cells were exposed to 25 μM doxorubicin and were harvested at 4 h intervals. Lysates were subjected to SDS-PAGE, followed by western blot analysis with the antibodies as indicated. (B) HaCaT cells were exposed to 25 μM doxorubicin. Eight hours later, 5 μM MG-132 or vehicle was added to the corresponding wells. Treatment continued for an additional 12 hours, at which time cells were harvested. Lysates were subjected to SDS-PAGE, followed by western blot analysis with the antibodies as indicated. (C) Western blot analysis with ZAK antibody on cell lysates from **Figure 3A**. (D) Western blot analysis with ZAK antibody on cell lysates from **Figure 3C**.

To determine if the multi-kinase inhibitors, sorafenib and nilotinib, could prevent the doxorubicin-induced changes in ZAK, we pretreated HaCaT cells with sorafenib or nilotinib (1 μM) followed by doxorubicin (10 or 25 μM) for 24 h. The presence of either inhibitor prevented both the disappearance of ZAK-α and the appearance of the higher molecular weight bands above ZAK-β, suggesting that the degradation of ZAK-α and the appearance of higher molecular weight bands above ZAK-β are coupled to the activation of ZAK (**Fig. 5C**). To determine whether suppressing the phosphorylation of JNK or p38 MAPK would inhibit doxorubicin-induced degradation or modification of the ZAK isoforms, we administered SB 203580 (10 μM), SP 600125 (20 μM), or a combination of the two to HaCaT cells 30 min prior to treatment with 25 μM doxorubicin for 24 h. The presence of either inhibitor or a combination of both did not prevent the disappearance of ZAK-α or the appearance of the higher molecular weight bands albove ZAK-β (**Fig. 5D**), suggesting that the ZAK-mediated activation of JNK or p38 MAPK did not act retrogradely to cause the disappearance of ZAK-α or the appearance of the higher molecular weight bands above ZAK-β.
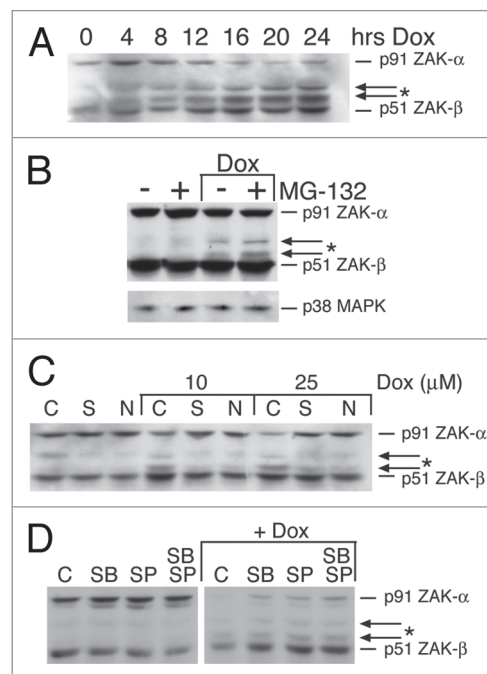
Discussion

In an effort to identify initial cellular targets of stressors, we uncovered a novel stress signaling pathway termed "ribotoxic stress", which results from the inhibition of protein synthesis due to interaction of the translational apparatus with disparate compounds such as antibiotics (anisomycin, blasticidin S, gougerotin), toxins (ricin, Shiga toxin, sarcin, palytoxin, tricothecene mycotoxins) and ultraviolet radiation. Transduction of signals that lead to activation of SAPKs occurs immediately upon exposure to these stressors and requires that the ribosome be actively engaged in protein synthesis at the time of exposure.[15,16,23,27,28]

Employing siRNA knockdown and chemical inhibition of ZAK, a MAP3K, Jandhyala et al. demonstrated that ZAK was required for ricin and Shiga toxin to mediate the activation of SAPKs and proinflammatory gene expression.[18] ZAK is one of 7 known mixed lineage kinases (MLKs) whose actions have been shown to mediate the activation of JNK and p38 MAPK.[29] An earlier study had demonstrated that siRNA-mediated knockdown of ZAK suppressed the activation of JNK and p38 MAPK by anisomycin and ultraviolet radiation, two other ribotoxic stressors.[17] Taken together, these studies suggest that ZAK uniquely communicates signals between ribosomes and the SAPKs.

The intercalation of doxorubicin and daunorubicin into DNA could comprise a major mode of anthracycline-induced cell death induced by these chemotherapeutics. Because doxorubicin also causes RNA damage[10] and inhibits DNA and RNA synthesis,[11,12] it is not unexpected that doxorubicin would also inhibit the synthesis of proteins.[13,14] In addition to inhibition of protein translation, doxorubicin induces the activation of SAPKs in a number of normal cell types, including hepatocytes,[6] primary mouse macrophages[7] and cardiomyocytes.[8,9] Our work presented here demonstrates that doxorubicin inhibits protein synthesis (**Fig. 1B**) and activates SAPKs (**Fig. 1A**), which suggests that doxorubicin, may act as a ribotoxic stressor and transmit signals through activation of ZAK.

We have employed clinically relevant doses of doxorubicin, ranging from 1–10 µM. HaCaT cells exposed to doxorubicin concentrations that are 2.5 µM or greater resulted in a progressive decrease in the incorporation of [^{3}H]-leucine over 24 h, suggesting that doxorubicin causes inhibition of translation (**Fig. 1B**). Cells treated with higher concentrations (10 and 25 µM) of doxorubicin responded with decreased levels of [^{3}H]-leucine incorporation to less than 10%, 24 h later. Doxorubicin also induced the phosphorylation of p38 MAPK and JNK when examined 24 h after addition of 5 to 50 µM doxorubicin. Knockdown of ZAK with siRNA abrogated the doxorubicin-induced phosphorylation of JNK and p38 MAPK (**Fig. 1A**), suggesting that ZAK was required for doxorubicin-induced activation of SAPKs. Taken together, these results demonstrated that doxorubicin behaves as a characteristic ribotoxic stressor by activating p38 MAPK and JNK through the upstream activation of ZAK.

SAPKs and NFκB participate together in the increased expression of proinflammatory cytokines.[30-35] Patients undergoing cancer chemotherapy display many of the classic symptoms of sickness behavior caused by the increased expression of cytokines, including IL-1β, TNFα and IL-6. Some of the acute side effects that accompany administration of chemotherapeutics include fatigue, nausea/vomiting, pain, sleep disturbances, cachexia and depression.[4] A life-threatening adverse reaction to doxorubicin treatment is cardiotoxicity, which is a serious limiting factor in the clinical use of doxorubicin.[3] Preclinical studies indicate that inflammatory responses may be involved in doxorubicin-induced apoptosis of cardiomyocytes.[36] For example, treatment with soluble Fas, an inhibitor of Fas/Fas ligand interaction that can lead to apoptosis, prevents doxorubicin-induced cardiotoxicity and concurrently attenuates the inflammation in cardiomyocytes.[37]

Pretreatment with statins can attenuate doxorubicin-induced cardiotoxicity via anti-inflammatory effects.[38] Olson et al. reported a novel anthracycline analog, DIDOX, which was structurally modified from doxorubicin. DIDOX inhibits the production of the pro-inflammatory cytokines, TNFα and IL-2. Studies in animal models show that, compared to doxorubicin, DIDOX inhibits inflammation and reduces cardiotoxicity.[39] Identification of agents that could selectively suppress the doxorubicin-induced inflammation in heart and other organs may prevent the death of non-tumor cells permitting the administration of larger doses of doxorubicin to cancer patients.

Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin.[8,9] Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line.[7] Employing inhibitors of p38 MAPK, JNK or ZAK we have asked whether activation of SAPKs would contribute to the doxorubicin-induced inflammation and apoptosis of non-tumor cells. Our findings that siRNA-mediated knockdown of ZAK suppressed the doxorubicin-induced apoptosis in HaCaT cells, as demonstrated by the reduction in cleavage of PARP and caspase-3 (**Fig. 1A**), is consistent with the role of ZAK acting through JNK and p38 MAPK to induce apoptotic death.

Previous studies have demonstrated that inhibition of ZAK by an experimental small molecule inhibitor (DHP-2; Eli Lilly) reduces ribotoxic stressor-induced cell death.[17,18] To further demonstrate the role of ZAK in doxorubicin-induced apoptosis of normal cells we employed two multi-kinase inhibitors with high affinity for ZAK, sorafenib (Bayer, K_d = 6.3 nM) and nilotinib (Novartis, K_d = 3 nM).[24,26] Nilotinib was developed as a second-generation inhibitor of BCR-ABL and has been successful in treating chronic myelogenous leukemia (CML) in patients that have developed resistance to imatinib (Gleevec). Nilotinib's binding affinity for ZAK is higher than its affinity for BCR-ABL.[40-42] Neither of these inhibitors had been tested for their ability to block ZAK activity in vitro. We demonstrated that sorafenib and nilotinib were each as effective as siRNA-mediated ZAK knockdown (**Fig. 3**), suggesting that these inhibitors can suppress the signaling pathway initiated by ZAK. In HaCaT cells, a pseudo-normal cell line derived from keratinocytes, sorafenib and nilotinib blocked doxorubicin- and duanorubicin-induced apoptosis and the phosphorylation of SAPKs (**Fig. 3A and B**). The suppression of JNK or p38-MAPK by the kinase inhibitors SP 600125 and/or SB 203580 showed partial protection against doxorubicin-induced apoptosis. However, the inhibition of apoptosis by these inhibitors was not as complete as sorafenib or nilotinib (**Fig. 3C**).

HeLa cells were more sensitive than HaCaT cells to the pro-apoptotic effects of doxorubicin. In contrast to the results in HaCaT cells, both sorafenib and nilotinib were unable to block doxorubicin-induced apoptosis in HeLa cells (**Fig. 4A**). We confirmed the role of ZAK in cytotoxicity following doxorubicin treatment by employing siRNA knockdown of ZAK (**Fig. 4B**). The inability of ZAK inhibition to suppress the pro-apoptotic actions of doxorubicin in HeLa cells, in contrast to HaCaT cells, suggests that pathways other than ZAK may play a role in cytotoxicity, in these cells, after doxorubicin treatment. The differential sensitivity of normal and cancer cells to the pro-apoptotic actions of doxorubicin suggest that inhibitors of ZAK may be effective in protection of normal cells against the cytotoxic activities of doxorubicin. However, this possibility must await further studies in an animal model.

ZAK has two different isoforms, ZAK-α (91 kDa) and ZAK-β (51 kDa). The two isoforms have identical protein kinase domains, including the ATP binding site, and separate functions for the two have not been defined.[18] HaCaT or HeLa cells treated with doxorubicin and immunoblotted for ZAK displayed

a progressive decrease in the ZAK-α band and the appearance of higher molecular weight bands above ZAK-β (**Figs. 1A and 4B**). Abrogation of these changes after exposure of the cells to sorafenib and nilotinib suggests that these changes occur following stimulation of ZAK by upstream signaling pathways. Degradation of ZAK following its activation suggests a homeostatic mechanism to suppress the continued activation of SAPKs by ZAK. Pretreatment of cells with the p38 MAPK inhibitor SB 203580, the JNK inhibitor SP 600125, or a combination of the two failed to prevent the doxorubicin-induced protein changes in ZAK, suggesting that activation of p38 MAPK or JNK are not involved in targeting ZAK for degradation.

We utilized MG-132, an inhibitor of proteasomal degradation, to determine if the doxorubicin-induced alterations in the two ZAK isoforms could result from ubiquitin-mediated proteolysis. The disappearance of the 91 kDa ZAK-α band was not prevented by the presence of MG-132, suggesting that it was not proteasome dependent. By contrast, the higher molecular weight bands above ZAK-β accumulated in the presence of the MG-132 compound, suggesting that these bands may represent ubiquitinylated forms of ZAK-β.

Sorafenib and nilotinib are in clinical use and exhibit very few side effects in patients. We suggest that these inhibitors could be employed in combination with doxorubicin to treat cancer patients because our data suggests that sorafenib or nilotinib might be able to reduce doxorubicin-induced apoptosis and SAPK phosphorylation in normal tissues. However, it is unknown if the presence of sorafenib or nilotinib in combination with doxorubicin could effect the antitumor activity of doxorubicin. Further experiments in animal models would determine the efficacy of treatment with doxorubicin and either sorafenib or nilotinib.

Materials and Methods

Antibodies and other reagents. Antibodies against the phosphorylated forms of ERK, JNK, p38 and against active caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against PARP (H-250), JNK1 (FL), and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Doxorubicin, daunorubicin, bisbenzimide, $CdCl_2$, and emetine were purchased from Sigma (St. Louis, MO). SB 203580, SP 600125, MG-132 and zVAD-fmk were purchased from EMD Chemicals (Gibbstown, NJ). Scrambled siRNA oligonucleotides (sequence: CAA CAC GGA CAU CAU ACG ATT), ZAK siRNA oligonucleotides (sequence from Wang et al.[17]), and ZAK#2 siRNA oligonucleotides (sequence: UGU UCA ACU CCU AAC UGC GTT) were synthesized by the Research Core Facility at Oregon Health & Science University, Portland, OR. Sorafenib and nilotinib were purchased from LC Laboratories (Woburn, MA). [3H]-leucine was purchased from PerkinElmer (Waltham, MA). ZAK antibody was kindly provided by Cheleste M. Thorpe (Tufts-New England Medical Center).

siRNA knockdown of ZAK. HaCaT or HeLa cells were grown to 70% confluency and treated in Dulbecco modified Eagle medium/10% FBS. ZAK or scrambled (scr) control siRNA were transfected, after titration of siRNA concentration

was performed, at 50 nM siRNA per well using DharmaFECT 4 (Dharmacon, Lafayette, CO) according to the manufacturer's instructions. After 72 h (HaCaT) or 48 h (HeLa), complete medium was removed and the cells were serum deprived for 1 h prior to addition of doxorubicin or vehicle (deionized water) for 24 h, at the indicated doses, prior to the harvest of cell lysates. Cell lysates were subjected to immunoblotting.

Fluorescent nuclear staining. HaCaT cells were plated on coverslips and exposed to siRNA for 72 hours as previously described, followed by exposure to 25 μM doxorubicin or vehicle (deionized water) for 24 h. Cells were fixed in 100% methanol for 5 min at 4°C and exposed to 10 μg/mL bisbenzimide. Images were captured by a Leica DFC350 FX camera attached to a Leica DM IRE2 microscope.

Measurement of protein synthesis via [3H]-leucine incorporation. HaCaT cells were grown in 12-well tissue culture plates. Treatments were performed in leucine-free/serum-free DMEM, for the indicated times and doses of doxorubicin. For the final 30 minutes of doxorubicin exposure prior to harvesting, cells were pulse-labeled with 1 μCi [3H]-leucine in 1 ml DMEM. Ten percent trichloroacetic acid was added to terminate incorporation. Following thorough rinsing with water, 88% formic acid was added to solubilize the trichloroacetic acid-insoluble proteins and the samples were counted in a liquid scintillation counter. In each experiment, three wells were used per experimental point.

Immunoblotting. Equal numbers of HaCaT or HeLa cells were plated, serum deprived for 30 minutes, treated, and lysed in 2XESB lysis buffer in preparation for immunoblotting. Equal volumes of the cell lysates were separated on a 10% or 13% (for caspase-3 antibody) denaturing polyacrylamide gel in the presence of sodium dodecyl sulfate and were transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies; signals were detected by using enhanced chemiluminescence.

Experiments with emetine. HaCaT cells were serum deprived and then pretreated for 0.5 h with 100 μg/ml emetine or vehicle (DMSO), followed by treatment with 0.5 mM doxorubicin, 100 μM $CdCl_2$, or vehicle (deionized water) for the indicated times. Cells were harvested at various times and subjected to immunoblotting.

Treatment with inhibitors. Serum-deprived HaCaT or HeLa cells were pretreated for 0.5 hours with sorafenib (1 μM), nilotinib (1 μM), SB 203580 (10 μM), SP 600125 (20 μM), zVAD-fmk (25 μM) or vehicle (DMSO). At 12 h an additional dose of equal amount was added to each well. Cells were treated with the indicated doses of doxorubicin (10 or 25 μM), daunorubicin (2.5, 10 or 25 μM), or vehicle (deionized water) for 24 h, harvested, and subjected to immunoblotting.

Doxorubicin time course. HaCaT cells were serum deprived and treated with 25 μM doxorubicin or vehicle (deionized water) in four-hour increments from 4–24 h. Cell lysates were subjected to immunoblotting.

Treatment with MG-132, a proteosome inhibitor. HaCaT cells were serum deprived and treated with 25 μM of doxorubicin

or vehicle (deionized water). Eight hours after addition of doxorubicin, 5 μM MG-132 or vehicle (DMSO) was added to the corresponding wells. Treatment continued for 12 hours for a total of 20 hours of doxorubicin treatment then cells were harvested and subjected to immunoblotting.

References

1. Takemura G, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Prog Cardiovasc Dis 2007; 49:330-52.

2. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 1999; 57:727-41.

3. Singal PK, Li T, Kumar D, Danelisen I, Iliskovic N. Adriamycin-induced heart failure: mechanism and modulation. Mol Cell Biochem 2000; 207:77-86.

4. Wood LJ, Nail LM, Gilster A, Winters KA, Elsea CR. Cancer chemotherapy-related symptoms: evidence to suggest a role for proinflammatory cytokines. Oncol Nurs Forum 2006; 33:535-42.

5. Kalyanaraman B, Joseph J, Kalivendi S, Wang S, Konorev E, Kotamraju S. Doxorubicin-induced apoptosis: implications in cardiotoxicity. Mol Cell Biochem 2002; 234:119-24.

6. Navarro R, Martinez R, Busnadiego I, Ruiz-Larrea MB, Ruiz-Sanz JI. Doxorubicin-induced MAPK activation in hepatocyte cultures is independent of oxidant damage. Ann NY Acad Sci 2006; 1090:408-18.

7. Elsea CR, Roberts DA, Druker BJ, Wood LJ. Inhibition of p38 MAPK suppresses inflammatory cytokine induction by etoposide, 5-fluorouracil, and doxorubicin without affecting tumoricidal activity. PLoS One 2008; 3:2355.

8. Kang YJ, Zhou ZX, Wang GW, Buridi A, Klein JB. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. J Biol Chem 2000; 275:13690-8.

9. Zhu W, Zou Y, Aikawa R, Harada K, Kudoh S, Uozumi H, et al. MAPK superfamily plays an important role in daunomycin-induced apoptosis of cardiac myocytes. Circulation 1999; 100:2100-7.

10. Fimognari C, Sestili P, Lenzi M, Cantelli-Forti G, Hrelia P. Protective effect of creatine against RNA damage. Mutat Res 2009; 670:59-67.

11. Wallace KB. Doxorubicin-induced cardiac mitochondrionopathy. Pharmacol Toxicol 2003; 93:105-15.

12. Momparler RL, Karon M, Siegel SE, Avila F. Effect of adriamycin on DNA RNA, and protein synthesis in cell-free systems and intact cells. Cancer Res 1976; 36:2891-5.

13. Nishida J, Shiratsuchi A, Nadano D, Sato TA, Nakanishi Y. Structural change of ribosomes during apoptosis: degradation and externalization of ribosomal proteins in doxorubicin-treated Jurkat cells. J Biochem 2002; 131:485-93.

14. White SJ, Kasman LM, Kelly MM, Lu P, Spruill L, McDermott PJ, et al. Doxorubicin generates a proapoptotic phenotype by phosphorylation of elongation factor 2. Free Radic Biol Med 2007; 43:1313-21.

15. Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Chen SL, et al. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol 1997; 17:3373-81.

16. Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Magun BE. Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. J Biol Chem 1998; 273:15794-803.

17. Wang X, Mader MM, Toth JE, Yu X, Jin N, Campbell RM, et al. Complete inhibition of anisomycin and UV radiation but not cytokine induced JNK and p38 activation by an aryl-substituted dihydropyrrolopyrazole quinoline and mixed lineage kinase 7 small interfering RNA. J Biol Chem 2005; 280:19298-305.

18. Jandhyala DM, Ahluwalia A, Obrig T, Thorpe CM. ZAK: a MAP3Kinase that transduces Shiga toxin- and ricin-induced proinflammatory cytokine expression. Cell Microbiol 2008; 10:1468-77.

19. Zhang SH, Wang WQ, Wang JL. Protective effect of tetrahydroxystilbene glucoside on cardiotoxicity induced by doxorubicin in vitro and in vivo. Acta Pharmacol Sin 2009; 30:1479-87.

20. Patel N, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-x$_L$ and p53 expression while preventing apoptotic and necrotic cell death in the liver. Toxicol Appl Pharmacol 2010; 245:143-52..

21. Batrakova EV, Li S, Brynskikh AM, Sharma AK, Li Y, Boska M, et al. Effects of pluronic and doxorubicin on drug uptake, cellular metabolism, apoptosis and tumor inhibition in animal models of MDR cancers. J Control Release 2010; 143:290-301.

22. Xu KH, Lu DP. Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukemia cells in vivo. Leuk Res 2009 2010; 34:658-65.

23. Iordanov MS, Magun BE. Different mechanisms of c-Jun NH(2)-terminal kinase-1 (JNK1) activation by ultraviolet-B radiation and by oxidative stressors. J Biol Chem 1999; 274:25801-6.

24. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, et al. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008; 26:127-32.

25. Keating GM, Santoro A. Sorafenib: a review of its use in advanced hepatocellular carcinoma. Drugs 2009; 69:223-40.

26. Manley PW, Drueckes P, Fendrich G, Furet P, Liebetanz J, Martiny-Baron G, et al. Extended kinase profile and properties of the protein kinase inhibitor nilotinib. Biochim Biophys Acta 2010; 1804:445-53.

27. Iordanov MS, Magun BE. Loss of cellular K⁺ mimics ribotoxic stress. Inhibition of protein synthesis and activation of the stress kinases SEK1/MKK4, stress-activated protein kinase/c-Jun NH2-terminal kinase 1, and p38/HOG1 by palytoxin. J Biol Chem 1998; 273:3528-34.

28. Iordanov MS, Choi RJ, Ryabinina OP, Dinh TH, Bright RK, Magun BE. The UV (Ribotoxic) stress response of human keratinocytes involves the unexpected uncoupling of the Ras-extracellular signal-regulated kinase signaling cascade from the activated epidermal growth factor receptor. Mol Cell Biol 2002; 22:5380-94.

29. Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol 2002; 3:663-72.

30. Pettus LH, Wurz RP. Small molecule p38 MAP kinase inhibitors for the treatment of inflammatory diseases: novel structures and developments during 2006–2008. Curr Top Med Chem 2008; 8:1452-67.

31. Uwe S. Anti-inflammatory interventions of NFkappaB signaling: potential applications and risks. Biochem Pharmacol 2008; 75:1567-79.

32. Sarkar FH, Li Y, Wang Z, Kong D. NFkappaB signaling pathway and its therapeutic implications in human diseases. Int Rev Immunol 2008; 27:293-319.

33. Schindler JF, Monahan JB, Smith WG. p38 pathway kinases as anti-inflammatory drug targets. J Dent Res 2007; 86:800-11.

34. Salh B. c-Jun N-terminal kinases as potential therapeutic targets. Expert Opin Ther Targets 2007; 11:1339-53.

35. Bogoyevitch MA, Arthur PG. Inhibitors of c-Jun N-terminal kinases: JuNK no more? Biochim Biophys Acta 2008; 1784:76-93.

36. Bruynzeel AM, Abou El Hassan MA, Schalkwijk C, Berkhof J, Bast A, Niessen HW, et al. Anti-inflammatory agents and monoHER protect against DOX-induced cardiotoxicity and accumulation of CML in mice. Br J Cancer 2007; 96:937-43.

37. Miyata S, Takemura G, Kosai K, Takahashi T, Esaki M, Li L, et al. Anti-Fas gene therapy prevents doxorubicin-induced acute cardiotoxicity through mechanisms independent of apoptosis. Am J Pathol; 176:687-98.

38. Riad A, Bien S, Westermann D, Becher PM, Loya K, Landmesser U, et al. Pretreatment with statin attenuates the cardiotoxicity of Doxorubicin in mice. Cancer Res 2009; 69:695-9.

39. Olson RD, Headley MB, Hodzic A, Walsh GM, Wingett DG. In vitro and in vivo immunosuppressive activity of a novel anthracycline, 13-deoxy, 5-iminodoxorubicin. Int Immunopharmacol 2007; 7:734-43.

40. Deininger MW. Nilotinib. Clin Cancer Res 2008; 14:4027-31.

41. O'Hare T, Eide CA, Deininger MW. New Bcr-Abl inhibitors in chronic myeloid leukemia: keeping resistance in check. Expert Opin Investig Drugs 2008; 17:865-78.

42. Bradeen HA, Eide CA, O'Hare T, Johnson KJ, Willis SG, Lee FY, et al. Comparison of imatinib mesylate, dasatinib (BMS-354825), and nilotinib (AMN107) in an N-ethyl-N-nitrosourea (ENU)-based mutagenesis screen: high efficacy of drug combinations. Blood 2006; 108:2332-8.