Akt, MAPK (Erk1/2), and p38 Act in Concert to Promote Apoptosis in Response to ErbB Receptor Family Inhibition*

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The ErbB receptor family is implicated in the malignant transformation of several tumor types and is overexpressed frequently in breast, ovarian, and other tumors. The mechanism by which CI-1033 and gemicabine, either singly or in combination, kill tumor cells was examined in two breast lines, MDA-MB-453 and BT474; both overexpress the ErbB-2 receptor. CI-1033, a potent inhibitor of the ErbB family of receptor tyrosine kinases, reduced levels of activated Akt in MDA-MB-453 cells. This effect alone, however, did not induce apoptosis in these cells. Gemicabine treatment resulted in a moderate increase in the percentage of apoptotic cells that was accompanied by activation of p38 and MAPK (ERK1/2). CI-1033 given 24 h after gemicabine produced a significant increase in the apoptotic fraction over treatment with either drug alone. During the combined treatment p38 remained activated, whereas Akt and activated MAPK were suppressed. Substitution of CI-1033 with the phosphatidylinositol 3-kinase inhibitor LY294002 and the MAPK/ERK kinase inhibitor PD 098059 in combination with gemicabine produced the same results as the combination of CI-1033 and gemicabine. p38 suppression by SB203580 prevented the enhanced cell kill by CI-1033. In contrast to MDA-MB-453, BT474 cells exhibited activated p38 under unstressed conditions as well as activated Akt and MAPK. Treatment of BT474 cells with CI-1033 inhibited both the phosphorylation of Akt and MAPK and resulted in a 47% apoptotic fraction. Gemicabine did not cause apoptosis in the BT474 cells. These data indicate that suppression of Akt and MAPK in the presence of activated p38 results in cell death and a possible mechanism for the enhanced apoptosis produced by the combination of CI-1033 and gemicabine in MDA-MB-453 cells. Furthermore, tumors that depend on ErbB receptor signaling for survival and exhibit activated p38 in the basal state may be susceptible to apoptosis by CI-1033 as a single agent.

Signal transduction through the ErbB receptor family involves the Raf-MEK-MAPK kinase and the PI 3-kinase-Akt signaling pathways. These pathways are often activated simultaneously with apparently conflicting responses such as apoptosis, proliferation, growth arrest, differentiation, and senescence, depending on the cell type and the duration and strength of the stimulus (1). The cytosolic serine/threonine protein kinase PKB/Akt is considered the focal point of a survival pathway known to protect cells from apoptosis during cytokine and growth factor stimulation. Heregulin, a known ligand for ErbB3/4, has been reported to regulate Akt in breast cancer cells (2) and when activated by signaling through the epidermal growth factor receptor protects epithelial cells against Fas-induced apoptosis (3). Activated Akt also has been shown to inhibit the apoptotic effects of farnesyltransferase inhibitors (4) and delay the onset of p53-mediated transcriptionally dependent apoptosis (5). In addition, phosphorylation of Raf by Akt was shown to inhibit activation of the Raf-MEK-MAPK signaling pathway and shifted the cellular response in a human breast cancer cell line from cell cycle arrest to proliferation (6).

Recent evidence has pointed to a relationship between the Akt and MAPK survival pathways and the structurally similar stress-activated members of the MAPK family, which include the stress-activated protein kinase JNK and p38 pathways in terms of a balance between cell death and proliferation. The precise role that signaling through the stress pathways plays remains complex, however, and many times seems to result in conflicting responses. For example, inhibition of the MAPK has been shown to play a role in the activation of p38 and induces apoptosis in HeLa cells (7). However, in trigeminal neurinoma cells, inhibition of p38 reduced the activity of MAPK and Akt and induced apoptosis (8). Rat mesangial cells conversely are resistant to tumor necrosis factor α-induced apoptosis that seem to be associated with an early transient JNK activation (9).

The ErbB receptor family is considered an important target in the treatment of cancer. Overexpression of one or more of these receptors occurs frequently in human tumors and often correlates with poor prognosis and shorter survival time (10, 11). Clinical validation for employing this receptor family as a target in cancer treatment has been achieved recently through specific antibody therapies directed toward ErbB2 (12) or the epidermal growth factor receptor (13). Lately, there has been a significant effort to identify specific inhibitors of the ErbB family of tyrosine kinases (14). CI-1033 is a potent and specific irreversible inhibitor of the ErbB receptor family (15) and is currently in phase I clinical trials (Fig. 1). This agent efficiently inhibits all signal transduction mediated via epidermal growth factor or heregulin (16) and exhibits broad spectrum antitumor activity in several human tumor xenograft models (17). Cancer agents are used frequently in combinations in the clinic, and recently protein kinase inhibitors have been shown to enhance the therapeutic effect of more conventional cytotoxic therapies (18). The present study examines the molecular mechanism by which CI-1033 induces apoptosis either as a single agent or in combination with gemicabine (2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluorodeoxyctydine, 2’2’-difluoro...
dFdC), an analog of deoxycytidine, with clinical activity against several types of cancer (19). The results indicate that the apoptotic response to CI-1033 and gemcitabine is caused by the inhibition of the survival pathways, MAPK and Akt, in concert with the activation of the p38 stress pathway. These studies further suggest that those tumors where survival signals are mediated through the ErbB family of receptors and exhibit constitutively activated p38 may be susceptible to CI-1033 as a single agent.

**EXPERIMENTAL PROCEDURES**

**Reagents**—LY294002, SB203580, FTTC-phalloidin, RNase, and propidium iodide were purchased from Sigma. CI-1033 and PD 098059 were synthesized at Pfizer, and gemcitabine was from Lilly. The antibodies used for Western blotting, phospho-Akt (Ser-473), Akt, phospho-MAPK, MAPK, phospho-p38, and p38 were purchased from New England Biolabs (Beverly, MA), and p27 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cultured Cells**—The human breast carcinomas, MDA-MB-453 and BT474 cells, were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown as monolayers at 37 °C in 5% CO₂ in air in Dulbecco's modified Eagle's medium/F12, 50:50 (Life Technologies, Inc.) containing 10% fetal bovine serum (Sigma) and gentamicin (Chemicon, Temecula, CA). Representative blots from duplicate experiments are shown in Figs. 3 and 4.

**Western Blots**—Cells were grown in 100 × 20-mm dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) seeded at 1.5 × 10⁶ cells/dish and allowed to grow 24 h before treatment as described under “Results.” Media and cells were collected and centrifuged at 200 × g for 8 min. The supernatant was removed and the pellet was washed once in PBS. The cells were lysed in 1 ml of ice-cold lysis buffer (50 mm HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml 4-(2-aminoethyl)benzenesulfon- nyl fluoride hydrochloride, 1 mM dithiothreitol). The lysate was transferred to a microcentrifuge tube, allowed to sit for 15 min on ice, and centrifuged for 20 min at 10,000 × g at 4 °C. The supernatant was transferred to a clean microcentrifuge tube and stored at −80 °C. Protein concentrations were determined by the BCA protein assay (Pierce). The proteins were run on 4–20% Tris/glycine polyacrylamide gel electrophoresis gel (Novex, San Diego, CA). Proteins (20 μg) were heated to 100 °C for 5 min, and loaded onto a 4–20% Tris/glycine polyacrylamide gel electrophoresis gel (Novex, San Diego, CA). Proteins in the gel were transferred to nitrocellulose (Novex), and the membrane was blocked for 1 h in blocking buffer (5% bovine serum albumin, 50 mM Tris (pH 8.0), 0.15 mM NaCl, 0.1% Tween 20, 10 mM β-glycerol phosphate, 1 mM NaF, 0.1 mM sodium orthovanadate). The membrane was blotted for 24 h in blocking buffer at 4 °C with the primary antibody, washed once for 15 min and three times for 5 min in wash buffer (PBS, 0.05% Tween 20) at room temperature, and blotted for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) in blocking buffer. Blots were washed in wash buffer once for 15 min and three times for 5 min. Proteins were detected by ECL (Amer sham Pharmacia Biotech) on BioMax MR-1 film (Sigma). The membranes were stripped using the Re-blot Western blot recycling 1 kit (Chemicon, Temecula, CA). Representative blots from duplicate experiments are shown in Figs. 3 and 4.

**Apoptosis**—Several methods were used to measure apoptosis. Cell rounding and detachment were noted during the treatment of the cells, and chromatin condensation was observed with a 4,6-diamidino-2-phenylindole stain. Poly(ADP-ribose) polymerase cleavage was determined by Western blotting, and caspase activation was measured using a kit from BioVision, Inc. (Palo Alto, CA). Apoptosis was determined also by actin/DNA staining using a modification of the procedure reported by Endrezen et al. (21). Briefly, 1.5 × 10⁶ cells were collected and centrifuged at 200 × g for 8 min, the medium was removed, and the cells were fixed by adding 3 ml of ice-cold 70% EtOH while gently vortexing. After a minimum of 1 h the cells were centrifuged at 200 × g for 8 min at 10 °C and the EtOH was aspirated. The actin was stained by resuspending the pellet in 20 μl of 10 μg/ml FITC-phalloidin in PBS (Sigma) in the dark at room temperature and washing once with PBS. The DNA was stained with 500 μl of propidium iodide (50 μg/ml in PBS) and 25 μl of RNase (600 kunitz units/ml) for 30 min at 37 °C in the dark. In the original protocol, the cells were fixed in paraformaldehyde; this was interfering with the propidium iodide staining so we used 70% EtOH, which prevented us from determining what phase of the cell cycle the cells were apoptosing from. The fluorescence was measured on a Coulter E-flow flow cytometer and analyzed using Winlist (Verity Software House, Inc., Topsham, ME). This method is based on the knowledge that actin is cleaved when cells enter apoptosis (22). All of the methods used to determine apoptosis gave the same apoptotic profile in each of the treatment regimens (data not shown). The apoptotic data presented here were obtained using actin/DNA staining.

**RESULTS**—In experiments involving treatment of MDA-MB-453 and BT474 cells, CI-1033 (0.5 μM in H₂O) and gemcitabine (50 μM in saline) were added as single agents or in combination for 3 days. The treatment protocols for the combination studies spanned 3 days and included 1) simultaneous addition, 2) addition of gemcitabine 24 h after CI-1033, and 3) addition of CI-1033 24 h after gemcitabine. LY294002 (10 μM), a PI 3-kinase inhibitor, and PD 098059 (1 μM), an MEK inhibitor, were made up in Me₂SO and substituted for CI-1033. When cells were pretreated for 24 h with SB203580 (10 μM in Me₂SO), a p38 inhibitor, experiments spanned 4 days. All experiments were done in duplicate.

The ability of CI-1033 to induce apoptosis either alone or in combination with gemcitabine was examined in two human breast carcinomas, the MDA-MB-453 and the BT-474. Both tumors express high levels of ErbB2; however, each exhibited markedly different responses to treatment with the two drugs. CI-1033 alone did not induce apoptosis in the MDA-MB-453 cells, whereas ~45% of the cell population was apoptotic in the presence of gemcitabine under the conditions described in Fig. 2. When these cells were exposed to CI-1033 and gemcitabine simultaneously, there was a significant enhancement of apoptosis and an even greater effect (82%) if the cells were treated sequentially first with gemcitabine and then CI-1033. This enhanced effect was not seen if cells were treated first with CI-1033 and then gemcitabine (Fig. 2), and in fact this schedule reduced the apoptotic response below that seen for gemcitabine alone. These effects were in sharp contrast to those obtained in the BT-474 breast tumor model in which CI-1033 alone induced significant apoptosis (47%) but gemcitabine had no effect (Table I). Moreover, treatment of these cells with a combination of CI-1033 and gemcitabine, regardless of the sequence of addition, produced no enhanced cytotoxic response.

The biochemical mechanism by which CI-1033 alone or in combination with gemcitabine causes cells to enter apoptosis was explored. This was looked at in the context of the major signaling pathways associated with the ErbB family of receptors, as well as the potential contribution of stress-activated pathways and modulation of the WAP family of cyclin-dependent kinase inhibitors, p27 and p21 (23). ErbB family signaling mediated through the Ras/Raf/MAPK pathway and PI 3-kinase/Akt survival pathway has been well established (24), and recent studies indicate that this receptor system also signals via the ErbB2/Akt/MAPK pathway and the p38 pathway (25). Modulation of these pathways was investigated with regard to the molecular mechanism by which CI-1033 and gemcitabine synergize and induce cell death. CI-1033 alone significantly suppressed constitutively activated Akt, MAP kinase, and increased p27 expression in the MDA-MB-453 cells (Fig. 3). A, B, and D) These effects alone, however, did not induce apoptosis (Fig. 2). Gemcitabine (50 μM) as a single agent had no effect on Akt but increased the phosphorylation of p38 as well as the
total p38 protein expression (Fig. 3C) and also increased the level of activated MAP kinase (Fig. 3B). These effects were associated with 45% of the cell population being apoptotic (Fig. 2). CI-1033 in combination with gemcitabine inhibited Akt, prevented the increased activation of MAPK phosphorylation, and stimulated p38 phosphorylation in MDA-MB-453 cells (Fig. 3). Apoptosis was most prominent (82% (Fig. 2)) when CI-1033 was added 24 h after gemcitabine and was associated with maximum inhibition of Akt, decreased activation of MAPK by gemcitabine, and sustained activation of p38 by gemcitabine. An increase in apoptosis also occurred when gemcitabine and CI-1033 were added simultaneously but was 10% less than when gemcitabine was added 24 h before CI-1033. When the order was reversed and CI-1033 was added 24 h before gemcitabine, the fraction of cells that were apoptotic was only 36% (Fig. 2). Under this schedule no activation of the p38 occurred (Fig. 3C), and there was incomplete suppression of Akt (Fig. 3A). In addition p27 expression was not reduced to the levels seen when gemcitabine was given as a single agent, simultaneously, or 24 h prior to CI-1033 treatment (Fig. 3D). p21 levels did not change under any of the treatment conditions (data not shown).

In contrast to the MDA-MB-453 cells, the BT474 cells exhibited constitutively activated p38 as well as highly activated Akt and MAPK. CI-1033 inhibited Akt and MAPK phosphorylation within 30 min in the BT474 cells, which in this tumor model was associated with a 47% increase in apoptosis by 72 h (Fig. 4 and Table I). In further contrast to the MDA-MB-453 cells, treatment of BT474 cells with gemcitabine alone did not induce apoptosis, and no cytotoxic synergy was observed in combination studies with gemcitabine and CI-1033.

To provide evidence that the enhancement of gemcitabine-mediated apoptosis by CI-1033 in the MDA-MB-453 cells was mediated through inhibition of both the PI 3-kinase/Akt and MAPK pathways, CI-1033 was substituted with a specific PI 3-kinase inhibitor, LY294002, and a specific MEK inhibitor, PD098059. When both the PI 3-kinase inhibitor and the MEK inhibitor were used in place of CI-1033 in combination with gemcitabine, 1% apoptosis was recorded (CI-1033 + gemcitabine) (Fig. 3D). In contrast, when CI-1033 was used alone, 45% apoptosis was observed (Fig. 3B). The combination of LY294002 and PD098059 resulted in complete inhibition of Akt and MAPK phosphorylation in the BT474 cells, which was associated with 72% apoptosis (Fig. 4B). These results suggest that the enhancement of gemcitabine-mediated apoptosis by CI-1033 is mediated through inhibition of both the PI 3-kinase/Akt and MAPK pathways.
Gemcitabine, an apoptotic profile occurred similar to that seen with CI-1033 as a single agent or in combination with gemcitabine (Table II). These results indicate that inhibition of both Akt and MAPK is at least partially responsible for induction of apoptosis in combination with gemcitabine. The requirement that activation of the p38 pathway occur for maximum induction of apoptosis in the presence of the drugs was established through the use of the p38 inhibitor SB203580 (26). Preincubation of the MDA-MB-453 cells with SB203580 prevented nearly all apoptosis in the presence of gemcitabine alone and reduced the percentage of apoptotic cells treated with gemcitabine followed by CI-1033 from 82 to 24% (Fig. 5). The signaling profiles in the presence of CI-1033 and gemcitabine obtained from both cell lines imply that maximum induction of apoptosis depends on both the suppression of survival pathways, which signal through Akt and MAPK, and simultaneous activation of the stress pathway through p38.

**DISCUSSION**

The balance between cell survival and cell death is a complex issue, and there is considerable effort to understand how tumor cells regulate the decision points between these critical pathways. A significant number of clinically useful anticancer agents have been shown to induce apoptosis in tumor cells (27), and this process is believed to be a major component of their therapeutic mechanism. The pathways through which these agents trigger apoptosis in cancer cells reportedly have been varied and tend to depend on both the nature of the drug and the genetic background and origin of the tumor (28). The present study provides evidence that modulation of both survival and stress-activated pathways plays a role in the strong synergistic interaction between gemcitabine and CI-1033 in terms of enhanced apoptosis in tumor cells. CI-1033 is a specific inhibitor of the ErbB family of receptor tyrosine kinases (16), which has been shown to be a viable target in cancer chemotherapy (14). Inhibitors of both the kinase activity and neutralizing antibodies to the extracellular domain are currently in clinical trials with encouraging results (14, 15). Gemcitabine is a nucleoside analog that, after phosphorylation, is incorporated into DNA and is a clinically approved agent for pancreatic cancer (19).

Signal transduction through the ErbB family of receptors has been shown to be mediated through two major pathways resulting in activation of Akt and the MAP kinases (Erk1/2), which have both been associated with mitogenesis and cell survival (24). CI-1033 significantly reduced the constitutively activated components of both pathways in the MDA-MB-453 breast tumor; however, this effect alone did not induce cell death. Gemcitabine alone, in contrast, caused a considerable percentage of the cell population to commit to apoptosis, and this process is believed to be a major component of their therapeutic mechanism. The pathways through which these agents trigger apoptosis in cancer cells reportedly have been varied and tend to depend on both the nature of the drug and the genetic background and origin of the tumor (28). The present study provides evidence that modulation of both survival and stress-activated pathways plays a role in the strong synergistic interaction between gemcitabine and CI-1033 in terms of enhanced apoptosis in tumor cells. CI-1033 is a specific inhibitor of the ErbB family of receptor tyrosine kinases (16), which has been shown to be a viable target in cancer chemotherapy (14). Inhibitors of both the kinase activity and neutralizing antibodies to the extracellular domain are currently in clinical trials with encouraging results (14, 15). Gemcitabine is a nucleoside analog that, after phosphorylation, is incorporated into DNA and is a clinically approved agent for pancreatic cancer (19).

**FIG. 3.** Western blot analysis of Akt, MAPK, p27, and p38 modulation in MDA-MB-453 cells treated with CI-1033 and gemcitabine. MDA-MB-453 cells (1.5 × 10^6) were seeded into a 100-mm plate. Twenty-four hours later, gemcitabine (50 nM) and CI-1033 (0.5 μM) were added as single agents, in combination (simultaneously), or in combination 24 h apart for a total drug exposure time of 72 h for all treatment regimens. Cell lysates containing equal amounts of proteins were run on 4–20% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blotted with antibodies against the phosphorylated forms of Akt (Ser-473), MAPK, and p38. The blots were stripped and reprobed with antibodies against total protein for Akt, MAPK, and p38.

**FIG. 4.** Western blot analysis of Akt, MAPK, and p38 modulation in BT474 cells treated with CI-1033. BT474 cells (1.5 × 10^6) were seeded into 100-mm plates. Twenty-four hours later CI-1033 (0.5 μM) was added as the indicated time points. Cell lysates containing equal amounts of proteins were run on 4–20% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blotted with antibodies against Akt, Erk1/2, and p38. The blots were stripped and reprobed with antibodies against total protein for Akt, MAPK, and p38.
dence for the involvement of these particular pathways was obtained with inhibitors of the Akt and MAP kinase pathways, LY294002 and PD 098059, respectively, both of which together mimicked the action of CI-1033, and the apoptotic response could be tempered by the p38 inhibitor, SB203580. No activation of JNK was seen under any of the described treatments and is probably not essential for the induction of apoptosis in this cell (data not shown).

The notion that activated p38 is necessary for CI-1033 to induce apoptosis is consistent with the ability of this drug to stimulate apoptosis in the BT-474 human breast carcinoma as a single agent. This cell exhibits highly activated p38, Akt, and Erk1/2 in the basal state and thus mimics the conditions seen in the MDA-MB-453 cells in the presence of gemcitabine. The latter agent in this case did not induce apoptosis in the BT-474 model presumably because these cells have learned to survive in the presence of activated p38 and die only when their survival pathways are suppressed as when treated with CI-1033. The pattern whereby apoptosis occurs only when Akt and MAP kinase pathways are suppressed in the presence of activated p38 is also consistent with results from the order of addition experiments. When MDA-MB-453 cells were exposed to CI-1033 first and then to gemcitabine, the resulting percentage of cells in apoptosis was markedly lower than with exposure to gemcitabine alone. The diminished apoptotic effect is consistent with the requirement for p38 activation because this sequence of drug additions did not result in activation of this enzyme (Fig. 3C). CI-1033, when added first, may indirectly inhibit p38 activation by gemcitabine by suppressing signaling through the ErbB receptor family, which is consistent with reports that p38 can be activated in cells stimulated with heregulin (NDF), a ligand for ErbB3 and ErbB4 (25). Alternatively, MDA-MB-453 cells treated with CI-1033 show a decrease in S phase cells as measured by bromodeoxyuridine uptake (data not shown). Thus gemcitabine may require cells to be cycling to be effective; gemcitabine-treated cells that are pretreated with the p38 inhibitor (SB203580) arrest in S phase (Fig. 5), where normally they would eventually enter apoptosis. However, if the cells were blocked earlier in the cell cycle by CI-1033, which is consistent with the observed increase in p27 levels, this could prevent the cytotoxic effects of gemcitabine.

The results from this analysis are consistent with studies in other cell systems that point to a combination of p38 activation in the absence of one or more survival pathways as a necessary condition to promote apoptosis. Inhibition of the MAP kinase pathway in HeLa cells by the MEK inhibitor PD098059 in the absence of serum stimulated the p38 pathway and initiated apoptosis (7). Cell death could be abrogated by serum, which apparently turned on the PI 3-kinase/Akt pathway as was demonstrated by the use of specific inhibitors and transfection of dominant negative mutants. Similarly, serum deprivation in Rat1 fibroblasts or nerve growth factor withdrawal from PC12...
cells resulted in apoptosis, which depended on activation of the p38 pathway (30). These observations are also consistent with the hypersensitive response that the C-26 murine colon carcinoma exhibits to the MEK inhibitor, CI-1040. The C-26 have a highly activated p38 pathway in the basal state and were exceptionally sensitive to inhibition of the MAP kinase pathway by CI-1040 as determined by clonogenic assays (31).

The above results suggest that elimination of the survival pathways involving Akt and Erk1/2 by inhibition of signaling through the ErbB family of receptors per se may not be a lethal condition for tumors and indicate that activated p38 may be required for cells to commit to apoptosis. Therapeutically then, these studies imply that simple assessment of the basal activation state of the stress-activated and survival pathways could identify tumors that are sensitive to single-agent inhibitors of the ErbB family as well as other signaling pathways. Furthermore, these studies indicate that the synergistic interaction between gemcitabine and CI-1033 is not because of the intrinsic biochemical mechanism of gemcitabine but perhaps because of the resulting stress response in general. That concept then suggests that inhibitors of the ErbB family have the potential to produce an enhanced cytotoxic response with any compound or condition that stimulates p38 activation and may have implications in the choice of drugs, or combinations of drugs, as well as patient selection for clinical trials.

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