Protective role for c-Jun in the cellular response to DNA damage

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Running title: Active c-Jun mediates resistance to DNA-damaging agents

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ABSTRACT

c-Jun, a member of the AP-1 family of transcription factors, has been implicated in the regulation of many important biological processes including cell cycle progression, transformation, differentiation, and apoptosis. Accordingly, its expression and function are upregulated in response to diverse stimuli including mitogens and a wide range of stresses. Transcriptional activation of the c-Jun protein is dependent on its phosphorylation at Ser63 and Ser73, a process mediated by c-Jun N-terminal kinase (JNK). Active c-Jun is required for AP-1 transactivation and c-Jun-mediated transformation, but its role during stress remains unclear as both pro-apoptotic and pro-survival effects of c-Jun have been observed. Here we investigated the importance of c-Jun N-terminal phosphorylation in influencing the sensitivity of human T98G glioblastoma cells to a variety of cytotoxic agents. Stable expression of a nonphosphorylatable dominant negative protein c-Jun(S63A,S73A) markedly inhibited activation of AP-1-driven transcription and greatly increased the cytotoxic effects of DNA-damaging agents associated with enhanced apoptosis. However, the same cells expressing the mutant Jun protein did not differ from parental cells in their sensitivity to several non-DNA-damaging cytotoxic agents. Our results suggest that activated c-Jun serves a selective role in protecting human tumor cells from apoptosis induced by DNA damage.
INTRODUCTION

c-Jun, a prominent member of the AP-1 transcription family, has been implicated in the regulation of a wide range of biological processes including development, differentiation, transformation, and apoptosis (1-4). Its transcriptional activities are regulated by changes in the level of c-jun expression as well as posttranslational modifications of the c-Jun protein. In particular, the transactivation potential of c-Jun critically depends on its phosphorylation of amino acid residues Ser63 and Ser73 (5, 6) mediated by c-Jun N-terminal kinase (JNK1) (reviewed in (1)). Not surprisingly, both c-jun expression and phosphorylation of c-Jun protein are highly induced in response to environmental cues including mitogenic stimuli as well as various stresses (7, 8). While the growth regulatory functions of c-Jun have been firmly established, its role in regulating responses to stress is more controversial.

Following stress, both prolonged c-jun expression and activation of c-Jun have been correlated with apoptotic responses. However, the actual role of c-Jun in influencing cell survival is far from clear. A pro-apoptotic role for c-Jun was first suggested in neuronal cells, where inhibition of c-Jun activity was found to protect cells from nerve growth factor (NGF) withdrawal (9). Consistent with this finding, overexpression of c-Jun in 3T3 fibroblasts resulted in the induction of apoptosis (10). Additional studies utilizing strategies to either over- or under-express c-Jun protein, or block its phosphorylation, have provided evidence that c-Jun is an important mediator of apoptosis (reviewed in (11)) induced by various cytotoxic agents including some chemotherapeutic drugs (12) and alkylating agents (13). However, a growing number of studies employing similar strategies to modulate c-Jun expression/activity have suggested that elevated expression and/or activation of c-Jun enhances cell survival during certain conditions of stress. In particular, a protective influence of c-Jun against UVC-induced apoptosis was supported by a recent study employing c-jun<sup>−/−</sup> fibroblasts (14, 15).
The basis for the seemingly disparate roles for c-Jun in regulating cell survival are unclear, but could reflect several different factors. One of these is the origin of the cells utilized, which has ranged from primary embryonic fibroblasts to different tumor cell types. The requirement of c-Jun could differ in normal versus transformed cells, in tumor cells of different cell lineages, or in cells having undergone transformation via different mechanisms. A second factor contributing to the apparent discrepancies noted above is the particular approach used to inhibit c-Jun function: in some models c-Jun expression is completely eliminated (i.e., embryonal fibroblasts from c-jun knock-out mice), whereas in others, only c-Jun phosphorylation/activation is prevented (e.g., expression of transactivation-deficient mutant forms of c-Jun protein or perturbation of the signaling pathways leading to c-Jun activation) (16-18). Recent studies showing that expression of a nonphosphorylatable dominant negative c-Jun mutant, c-Jun(S63A,S73A) (5, 6), can partially rescue proliferation defects in c-jun−/− cells (18, 19) suggest that a deficit in phosphorylated c-Jun protein is not equivalent to the absence of c-jun expression (11). A third factor likely to be important in determining the influence c-Jun has on cell survival is the particular stress condition investigated. The extent of AP-1 activation can vary greatly from one treatment to another, and with different dosages of an agent or durations of exposure. In addition, parallel activation of other stress-responsive signaling pathways and/or genes could influence the relative importance of c-Jun for survival under a given condition.

Previous findings using several human tumor cell lines that continuously express high levels of nonphosphorylatable dominant negative c-Jun(S63A,S73A), referred to hereafter as dnJun, suggested a protective role for c-Jun N-terminal phosphorylation against cisplatin-induced cytotoxicity (20, 21). In the present study we sought to extend these earlier observations and therefore investigated the importance of c-Jun activity in determining the fate
of human T98G glioblastoma cells exposed to a variety of DNA-damaging as well as non-DNA-damaging cytostatic/cytotoxic compounds. We report that T98G cells expressing dnJun exhibit enhanced sensitivity to DNA-damaging treatments, but not to other stressors that act independent of DNA damage to inhibit growth and/or induce cell death. These findings suggest that N-terminus phosphorylation-dependent activation of c-Jun has an important role in protecting human tumor cells against DNA damage-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Treatments, and Viability Assay** - Human glioblastoma T98G cells and their dnJun derivatives were previously described (20). Cells were cultured in DMEM (Gibco BRL), supplemented with 10% fetal bovine serum (HyClone). To assess their sensitivity to various stresses, cells were seeded at a density of 1,000 cells/well in 96-well plates and on the following day were treated in the same medium (except in the case of UVC irradiation) with cytotoxic agents for 1 h. Following all treatments cells were washed with PBS and supplemented with fresh complete medium. In the case of UVC treatment, cells were washed with PBS prior to irradiation and fresh medium was added immediately after treatment. Measurements of viable cell mass were performed 5-7 days later using a colorimetric-based reaction following addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) in accordance with the manufacturer’s protocol (Promega). All determinations were carried out in triplicate, and cell viability was expressed as viable cell mass following a given treatment normalized to that of parallel cultures of untreated cells (Cell Viability, %). The chemicals used were purchased from Brystol Laboratories (Platinol®-AQ cisplatin injection), Sigma (etoposide, thapsigargin, tunicamycin,
tamoxifen, MNNG, taxol (paclitaxel), Aldrich Chemical Company (MMS), and Calbiochem (doxorubicin, daunorubicin, mitoxantrone, streptonigtin, actinomycin D).

**CAT assay** - Transient transfections were carried out using the standard calcium phosphate precipitation method as described (22). Briefly, T98G cells were seeded into 24-well tissue culture plates at a density of 5x10^3 cells/well and transfected the following day with 2 µg total plasmid DNAs per well (0.1 µg of MEKK1, 0.9 µg of expression vectors for either c-Jun or dnJun, and 1 µg of CAT reporter DNA) for 18 h. After washing with PBS containing 0.5 mM EGTA, fresh medium containing 0.5% FCS was added. Twenty four hours later the cells were lysed, mixed with CAT reaction mixture (1 mM chloramphenicol, 15 µM HCl, 0.4 µl of carrier-free [³H]-Acetate and 0.6 µl Sodium Acetate in 150 mM Tris HCl pH 7.5) and incubated at 37°C for 1 h. The samples were transferred into 1 ml of 7 M Urea, mixed by vigorous shaking with 1 ml of Toluene containing 8 g/L 2,5-Diphenyloxazole (Sigma), and CAT activity was measured by liquid-phase scintillation. All experimental points were carried out in triplicate, and shown are the mean values of four such experiments.

**In vitro kinase activity assay** - Cells were treated with various agents and lysates were prepared 30 min later in whole-cell extract buffer (25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 100 µg/ml PMSF, 0.1 mM Na₃VO₄, 20 mM a-glycerophosphate, 2 µg/ml leupeptin and 0.54 mM freshly added dithiothreitol). The JNK kinase assay was performed as described before (23) using sc-571 JNK antibodies (Santa Cruz Biotechnologies) for precipitation, and rat recombinant GST-c-Jun(1-79) protein (Biomol Research Laboratories) as substrate. The samples were resolved by electrophoresis through 12% polyacrylamide-SDS gels, and [γ-³²P]-phosphorylated GST-cJun was quantified by using a PhosphorImager (Molecular Dynamics).
**DNA synthesis assay** - DNA synthesis rates were measured using a $[^3\text{H}]$-Thymidine incorporation assay. Cells were seeded in 96-well tissue culture plates (1,000 cells/well) and treated with cisplatin for 1 h as described above. At designated times after the treatment, $[^3\text{H}]$-Thymidine (0.5 mCi/well) was added for 3 hours. Cells were harvested with a PhD-200A cell harvester (Cambridge Technologies), which transferred the labeled lysates onto paper disks. These disks were subsequently washed and the amount of radioactive DNA was quantitated by scintillation counting using Biosafe-II scintillation liquid.

**Flow Cytometric Cell Cycle Analysis** - Cell cycle distribution was analyzed using flow cytometry. Briefly, cells were trypsinized, washed with PBS, fixed in 70% ethanol, washed again in PBS, incubated with RNAse, and stained with PI (propidium iodide) using a Cellular DNA Flow Cytometry Analysis kit (Boehringer Mannheim). Data were acquired with 1024 channels of resolution on FACScan (Becton Dickinson) and percentages of cells in the various cell cycle stages were determined using Multicycle software (Phoenix).

**RESULTS**

*Expression of phosphorylation-deficient c-Jun(S63A,S73A) in T98G cells results in growth inhibition and reduced AP-1 transactivation* - To investigate the importance of c-Jun N-terminal phosphorylation in determining the sensitivity of human tumor cells to various stresses, T98G cells that overexpress a nonphosphorylatable dominant negative mutant c-Jun(S63A,S73A) protein, dnJun, were generated (20). The levels of c-Jun protein in two such dnJun cell lines (clones 10-6 and 10-10) as well as parental T98G cells transfected with empty vector (T98GX) are shown in Fig. 1A. The T98GdnJun clones showed approximately 5-6 fold higher amounts of total c-Jun protein (endogenous c-Jun and dnJun proteins combined) relative to T98GX cells, indicating a high level of dnJun expression. The expression of the dnJun in
T98G cells was associated with growth inhibition in clonal cell lines, characterized by slower rates of proliferation and reduced saturation densities (Fig. 1B).

The effect of dnJun on cell growth could result from at least two different mechanisms. Nonphosphorylatable dnJun could act as a dominant negative inhibitor of AP-1 transcriptional activity, as has been previously suggested (5, 6). Alternatively, given that JNK is proposed to bind tightly to c-Jun and release it only after phosphorylation (24), dnJun could block JNK activity directly by acting as a pseudo-substrate and tying up JNK protein. To explore the first possibility, the AP-1 transcriptional activity of T98G cells expressing dnJun was examined using transiently-transfected CAT-based reporter constructs linked to various AP-1 binding elements to which c-Jun has been shown to interact (25, 26). The JNK pathway was then specifically stimulated by expression of a constitutively active mutant form of the upstream kinase, MEKK1 (27). This resulted in strong activation of AP-1 (6xAP-1 and 3xTRE) and ATF/CREB (5xjun2) binding site reporter constructs (Fig. 1C, open bars) without affecting CAT expression driven by a control promoter 56FosΔE, which lacks a functional AP-1 site (22). Transient expression of dnJun (solid bars), but not wild type c-Jun (hatched bars), lead to a substantial inhibition (>80% decrease) of all three AP-1 reporter constructs used in these experiments (Fig. 1C). Thus, consistent with observations in 3T3 fibroblasts (5, 6, 18), MEKK1-mediated transcriptional transactivation of AP-1 in human T98G glioblastoma cells was dependent on the availability of active c-Jun.

To determine whether dnJun interfered directly with JNK activity, JNK activity was measured using an immunoprecipitation kinase assay with c-Jun (1-79) as a substrate in T98GX and T98dnJun cultures 30min after treatment with either 40 J/m² UVC or 200 µM cisplatin. Expression of dnJun did not affect the level of JNK activity following UVC (Fig. 1D, bottom panel) or cisplatin (data not shown) treatments suggesting that permanent
association of JNK and dnJun molecules did not occur. These results argue against a direct effect of dnJun on the JNK enzyme and indicate that expression of dnJun in T98G cells inhibits c-Jun-phosphorylation-dependent AP-1 transactivation consistent with a dominant negative mechanism.

Expression of phosphorylation-deficient dnJun sensitizes T98G cells to DNA-damaging agents, but not to other cytotoxic compounds - Assessment of long-term (5-7 days) growth effects in cells subjected to 1-h exposure to different cytotoxic compounds was performed using an MTS tetrazolium dye conversion assay measuring viable cell mass (Promega). A reduction in viable cell mass can occur as result of growth inhibition or cell death. A variety of agents known to act via different mechanisms to damage DNA were tested (Table 1). As shown in Fig. 2, cells harboring dnJun were more sensitive than their normal counterparts to the growth inhibitory effects of all of the DNA-damaging agents tested, regardless of the compound’s mode of action. Parental and dnJun-expressing T98G cells were also examined for their sensitivity to several other agents known to exert growth inhibitory or toxic effects on cells unrelated to DNA damage. These included the microtubule-disrupting agent taxol, the anti-estrogen agent tamoxifen, and two agents known to cause stress to the endoplasmic reticulum, thapsigargin and tunicamycin (Table 1). As seen in Fig. 3, no differences in sensitivity to any of these agents were seen when comparing the results for T98GX and T98GdnJun cells.

DNA synthesis, cell cycle progression and apoptosis in T98GX and T98GdnJun cells - A number of different mechanisms such as (i) impaired DNA synthesis, (ii) perturbations in cell cycle progression, and (iii) induction of apoptosis could contribute to the reduction in viable cell mass seen following treatment with cytotoxic agents. c-Jun activity could influence such effects thereby accounting for the differential sensitivity of T98GX and T9GdnJun cells.
(Figs. 2 and 3). To characterize contribution of various mechanisms to the enhanced loss of cell mass in T98GdnJun cultures following DNA-damaging treatments we explored the effects of a well-characterized chemotherapeutic agent cisplatin, on growth properties of T98GX vs T98GdnJun cells.

First, DNA synthesis was examined in T98G and T98GdnJun cultures treated with various doses of cisplatin and transplatin. While treatment of cells with transplatin, a platinum compound that does not induce DNA-damage responses, had no significant effect on DNA synthesis (data not shown), DNA synthesis was markedly inhibited in cisplatin-treated T98GX and T98GdnJun cells. This inhibition was dose-dependent and transient, as determined by tritiated thymidine incorporation at various time points (24-96 hours) (data for 24 h shown in Fig. 4A). However, when normalized to cell number, the degree of inhibition as well as the rates of recovery were similar in parental and dnJun-expressing cells (Fig. 4B), indicating that greater inhibition of DNA synthesis is not the cause for the enhanced growth inhibitory effects of DNA-damaging agents in T98GdnJun cells.

Morphological examination of cisplatin-treated T98GdnJun cells by phase contrast microscopy revealed features such as cell rounding and membrane blebbing, suggestive of apoptosis (Fig. 5). Further examination of the cells by DAPI staining revealed clear evidence of condensed and fragmented nuclei. These morphological features were much less apparent in T98GX cells treated with cisplatin, and were absent in thapsigargin-treated cells regardless of c-Jun status (Fig. 5), suggesting that the degree of apoptosis seen in cells treated with DNA-damaging agents can depend on c-Jun status. Therefore, we examined the cell cycle distribution of untreated and cisplatin-treated T98GX and T98GdnJun cells. Similar cell cycle profiles were seen for the T98GX and T98GdnJun cells under normal growth conditions (results for clone T98GdnJun10-10 are shown in Fig. 6A), although a small but significant (p <
0.02, Student’s t-test) difference in the proportion of cells in the G1 and S phases was noted (Fig. 6B). The greater proportion of T98GdnJun cells in G1 with a reduced number in the S phase compartment could reflect the slower growth rate of the T98GdnJun cells. Cisplatin treatment resulted in an increased accumulation of cells in S phase and loss of cells in the G2 compartment in both T98GdnJun and T98GX cultures. However, consistent with the morphological analysis above, cisplatin treatment of dnJun-expressing cells resulted in the appearance of a significant sub-G1 compartment that was much less apparent in T98GX cells (Fig. 6C, upper panel). A similar effect was noted following etoposide treatment (data not shown). Treatment of T98GX and T98GdnJun cells with thapsigargin resulted in a very different profile from that seen with cisplatin. Both parental T98GX and dnJun-expressing T98GdnJun cells became arrested in the G1 phase of the cell cycle with no accumulation of cells in sub-G1 region (Fig. 6C, lower panel).

Additional confirmation of the differential extent of apoptosis in T98GX and T98GdnJun cells following treatment with DNA-damaging agents was obtained using an ELISA-based assay that detects the presence of histone-associated DNA fragments in the cytoplasm. As shown in Fig. 6D for the T98GdnJun10-10 clone, all three of the DNA-damaging agents tested caused greater amounts of apoptosis in T98GdnJun cells relative to T98GX, while no differences in the levels of apoptotic signals were detected in T98GX and T98GdnJun cultures treated with taxol, thapsigargin or tunicamycin (data not shown).

In summary, both long-term viability and short-term apoptosis assays indicated that overexpression of phosphorylation-deficient dnJun protein renders human T98G glioblastoma cells much more sensitive to from DNA-damaging agents relative to their normal counterparts. These results support an important role for c-Jun in cellular protection against such insults.
DISCUSSION

Significant controversy exists in the published literature regarding the role of c-Jun in regulating cell survival during the stress response. Often, the mere correlation between enhanced expression of c-Jun and the onset of apoptosis has led authors to conclude that c-Jun is directly involved in mediating cell death, but in such instances c-Jun expression/activation could in fact occur as part of a failed defense response aimed at cell protection. More mechanistic studies in which either c-Jun expression or functional activity has been manipulated through gene targeting or mutagenesis strategies have also produced disparate findings. Such discrepancies could reflect the nature of the manipulations employed, the cell types studied, or the particular stress conditions evaluated. For example, both pro-survival and pro-apoptotic roles of c-Jun have been reported using c-jun−/− fibroblasts derived from knockout mice when different conditions were applied (13, 15). Whether pro-survival or pro-apoptotic, it is clear that a better understanding of the role c-Jun plays in determining cell survival under conditions of stress could lead to improved strategies for treatment of human cancers.

Here we investigated the role of activated c-Jun in influencing viability of human tumor cells subjected to various cytotoxic stresses. The model employed consisted of T98G glioblastoma cells engineered to express high levels of a c-Jun(S63A,S73A) protein in which serine residues, known to be critical for the phosphorylation-dependent transactivation of c-Jun-containing AP-1 complexes, were mutated to alanines (5, 6). Consistent with previous observations with a transactivation-deficient mutant form of c-Jun, TAM67 (28), replacement of endogenous c-Jun from AP-1 complexes by overexpression of a nonphosphorylatable dnJun protein led to a successful inhibition of AP-1 transactivation (Fig. 1C). It is interesting to note that overexpression of phosphorylation-deficient dnJun had no inhibitory effect on JNK activation under conditions of genotoxic stress (Fig. 1D) suggesting that the effects described
in the present study were solely due to inhibition of phosphorylation-dependent c-Jun-mediated functions while other targets of JNK signaling in stressed cells remained unaffected.

Examining the responsiveness of normal and dnJun-expressing T98G cells (T98GX and T98GdnJun, respectively) to different cytotoxic agents we observed no differences in their sensitivities to any of four compounds known to act independent of DNA damage to exert the cytotoxic effects (Fig. 3). This result is consistent with the previous observation that activation of the MEKK1 pathway has no role in the induction of apoptosis by microtubule altering drugs (16). However, T98GdnJun cells displayed much greater sensitivity to all of twelve different DNA-damaging agents tested (Fig. 2, data not shown). Interestingly, this effect was seen regardless of the agent’s particular mode of action; i.e., agents capable of causing single and double strand DNA breaks such as UVC (Fig. 2B) and γ-irradiation (data not shown), agents creating DNA adducts (cisplatin), alkylating agents (MMS, MNNG), topoisomerase inhibitors (etoposide), and agents that cause extensive oxidative damage (streptonigrin) (summarized in Table 1). This suggests that c-Jun or some other AP-1 component whose activity is subject to interference by the presence of the mutant c-Jun protein serves a general protection function during the response to DNA damage. These findings appear to contradict the more widely held view of c-Jun as a mediator of apoptosis (10-12). However, like ours, several previous studies have suggested a protective function for c-Jun following DNA damage. These include reports demonstrating that abrogation of c-Jun induction renders cells more sensitive to DNA damage (21, 29), and that elevated c-Jun expression render cells more resistant to doxorubicin-induced cell death (30).

One possible way in which c-Jun could influence cell survival during the response to DNA damage is through affecting DNA repair capacity. Indeed, in a previous study (20), we provided evidence suggesting that inhibition of the JNK pathway affects the ability of T98G
cells to repair cisplatin-generated DNA adducts (20). In association with the present study we examined the efficiency of DNA repair following treatment with 50-100 µg/ml etoposide in T98GX and T98GdnJun cells using a Comet assay (31). This single cell DNA repair assay allows assessment of DNA repair of double strand DNA (dsDNA) breaks. While one of the two T98GdnJun cell clones (clone 10-6) employed in the current studies exhibited reduced repair of etoposide-induced dsDNA breaks compared to control cultures 2 h following the treatment, the other T98GdnJun cell line did not (data not shown). Thus, it appears unlikely that differential repair capacity is the explanation for the enhanced sensitivity of T98GdnJun cells to DNA-damaging treatments. However, it is worth noting that although dsDNA breaks is the prevailing form, it is not the only type of DNA damage caused by etoposide, and it is conceivable that other repair activities could also influence the response. Human cells have multiple systems responsible for repair of various types of DNA damage and obviously more detailed investigations will be necessary to determine what, if any, relationship exists between c-Jun and DNA repair in influencing sensitivity of dnJun-expressing T98G cells to DNA damaging agents.

As reported here, T98GdnJun cells exhibit greater apoptotic changes following treatment with DNA-damaging treatments than do their wild type counterparts. Evidence of enhanced apoptosis in T98GdnJun cells following treatment with the DNA damaging agents included (i) general morphological appearance and detachment of cells from the tissue culture plates (Fig. 5, data not shown), (ii) higher levels of fragmented DNA as assessed in an ELISA-based assay (Fig. 6D); (iii) higher numbers of fragmented nuclei as visualized by DAPI staining (Fig. 5), and (iv) a greater proportion of cells in the sub-G1 compartment as determined by FACS analysis (Fig. 6C). No such differences were noted in cultures treated with non DNA-damaging agents. For example, treatment with thapsigargin resulted in a
marked G1-arrest in both T98GX and T98GdnJun cells (Fig. 6C). Such G1 arrest has been implicated as an important factor in the survival of thapsigargin-treated cells (32). Treatments with taxol and tamoxifen, on the other hand, resulted in significant apoptosis but levels were indistinguishable comparing T98G and T98G-dnJun cells (data not shown). Thus, the presence of active c-Jun and therefore availability of active c-Jun-containing AP-1 complexes appears to have a selective role in protecting cells from apoptosis induced by DNA damage.

The effects observed in the present study suggest that activated AP-1 complexes exert a favorable influence on cell survival following sensitivity to DNA damage. If so, then like the expression of nonactivatable c-Jun, inhibition of upstream regulators of c-Jun activation should result in sensitization of T98G cells to DNA-damaging agents. At the present time, no specific chemical inhibitors of c-Jun or JNK are available, but we have shown that expression and activation of JNK can be efficiently inhibited through the use of specific JNK antisense oligonucleotides (33). In preliminary studies we have found that, as seen for T98GdnJun-expressing cells, pretreatment of T98G cells with JNK antisense oligonucleotides enhances their susceptibility to cisplatin treatment (data not shown).

In summary, our findings suggest that activated c-Jun serves a selective function in protecting human tumor cells against cytotoxic effects of DNA damaging agents. The heightened sensitivity of cells lacking phosphorylation-dependent Jun functions could be exploited in the development of therapeutic regimens for the treatment of human cancers where combined use of DNA damaging chemotherapeutic agents with inhibitors of c-Jun activation could have clinical benefits.

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1 Abbreviations used: JNK, c-Jun N-terminal kinase; NGF, neuronal growth factor; AP-1, activation protein 1; dnJun, dominant negative c-Jun(S63A,S73A); MMS, methyl methanesulfonate; ROS, reactive oxygen species; DAPI, 4'-6-diamidino-2-phenylindole; MTS, (3-(4,5'-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt.; PI – propidium iodide; UVC, ultraviolet light C band.
FIGURE LEGENDS

Fig. 1. Characterization of dnJun effects in T98G cells. A, Total c-Jun expression levels in vector-transfected control cell line T98GX and 2 clonal isolates of T98G cells expressing mutant c-Jun protein c-Jun(S63A,S73A) (labeled T98GdnJun). Western blot was performed using sc-1694 c-Jun antibodies (Santa Cruz Biotechnologies). B, Growth properties of T98GdnJun clones (filled symbols) and T98GX cells (open circles). Cells were seeded at a density of 25x10^3 cells/cm^2, and counted daily in triplicate (Coulter counter). C, Expression of phosphorylation-deficient dnJun protein inhibits AP-1 transactivation in T98G cells. T98G cells were simultaneously transiently transfected with designated plasmid DNAs as described in the Experimental Procedures. Constitutively active MEKK1 was used to activate the JNK signaling pathway; 6xAP-1, 5xjun2 and 3xTRE are reporter constructs preferentially activated by various AP-1 heterodimers; 56FosΔE is a control, non-activatable reporter construct derived from AP-1 binding region of c-fos promoter used in 6xAP-1 construct. Shown are the mean values from four independent experiments (each carried out in triplicate). D, Expression of dnJun protein does not affect JNK activation following UVC irradiation of T98G cells. JNK activation was measured using an in vitro kinase activity assay as described in the Experimental Procedures. Similar low basal JNK activities (upper panel) as well as levels of JNK activation 30 min after exposure to 40 J/m^2 UVC (lower panel) were detected in the various T98G cell lines. The numbers on the bottom of the plot reflect relative levels of JNK activation in the various clones normalized to JNK activity in control T98G cells following UVC irradiation as determined by a PhosphorImager.

Fig. 2. Expression of phosphorylation-deficient dnJun protein results in enhanced sensitivity of T98G cells to various DNA-damaging agents. Cells were seeded at 1,000
cells/well density in 96-well tissue culture plates and on the following day were treated for 1 h with the designated agents. Viable cell mass was assessed 5-7 days later using an MTS dye assay, and is expressed as the percentage of the value obtained in a parallel untreated culture. All assays were carried out in triplicate and repeated at least four times (shown is a representative experiment for each treatment).

**Fig. 3. Viability of T98G cells following treatment with non DNA-damaging agents is not affected by expression of dnJun protein.** All assays were carried out as described in a legend for Fig. 2.

**Fig. 4. Inhibition and recovery of DNA synthesis in T98G and T98GdnJun cells following cisplatin treatment.** A, Cisplatin treatment results in a marked inhibition of DNA synthesis in all T98G cultures. Cells were seeded in 96-well plates at a density of 1,000 cells/well and on the following day exposed for 1 h to designated concentrations of cisplatin. DNA synthesis was assessed by \(^{3}\)H-Thymidine incorporation 24 h following the treatment as described in the Experimental Procedures. Values are normalized to number of viable cells as determined by an MTS assay at the same time in parallel cultures. The results are expressed as percentage of DNA synthesis in untreated control cultures of the same origin. Each treatment point was carried out in triplicate. B, Kinetics of recovery of DNA synthesis in T98G cells treated with cisplatin. Cells were treated for 1 h with various concentrations of cisplatin and DNA synthesis was determined at designated time points as described above.

**Fig. 5. DAPI staining of cisplatin- and thapsigargin-treated cells.** T98GX and T98GdnJun cells were seeded at a density of 5x10^4 cells/cm^2, and on the following day were treated with indicated agents. Twenty four h later they were fixed and stained with 1 µg/ml DAPI. Microphotographs were taken using phase-contrast (black and white figures) and fluorescent
microscopes (color figures) at 400 X magnification. The photographs shown were taken from
different fields of the same cultures before (phase-contrast) and after (fluorescent) DAPI
staining. Note the presence of condensed and fragmented nuclei in cisplatin-treated T98Gdn
cells.

**Fig. 6. Cell cycle and apoptosis analysis of T98G and T98GdnJun cells.** A, Cell cycle
analysis in normal-growing cultures. T98GX and T98GdnJun cells were seeded at a density of
5x10^4 cells/cm^2, maintained under normal growth conditions (10% FCS), and prepared for
flow cytometric analysis 48 h after being seeded. Data were acquired with 1024 channels of
resolution on FACScan (Becton Dickinson) (channels 0-450 are shown) and analyzed with
Multicycle software. B, Distribution of cells between various cell cycle compartments as
assessed by Multicycle software. Shown are mean values obtained from four independent
experiments (data obtained with T98GdnJun 10-6 and 10-10 clones were pooled for the
analysis). A statistically significant difference in the number of cells in S phase was detected
between T98GdnJun and T98G cultures (* p < 0.02, Student’s t-test). C, Flow cytometric
analysis of cisplatin- and thapsigargin-treated T98GX and T98GdnJun cells. Cells were seeded
at a density of 5x10^4 cells/cm^2 and treated the next day with either cisplatin (*upper panel*)
or thapsigargin (*lower panel*) for 24 h. PI staining was performed as described in the
Experimental Procedures. D, Biochemical assessment of apoptosis following DNA damage.
Cells were seeded at a density of 5x10^4 cells/cm^2, and treated the following day for 1 h with
either 100 µM cisplatin, 1 µg/ml doxorubicin, or 50 µg/ml etoposide. Apoptosis was assessed
24 h later using an ELISA-based assay that measures levels of degraded nuclear DNA in the
cytoplasm. The assay was performed as specified by the manufacturer (Boehringer
Mannheim) and each treatment point was assayed out in triplicate. The results are expressed as
percentage of values seen in untreated parallel cultures of the same origin.
Table 1. Treatments description.

| Cytotoxic Agent     | DNA Damage | Stress Description (types of DNA damage and other effects)                                                                                                                                 |
|---------------------|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cisplatin (cis-DDP) | Yes        | Introduces interstrand/intrastrand DNA crosslinks and DNA-protein crosslinks. Cell cycle block (G1/S and G2), apoptosis.                                                                  |
| MMS                 | Yes        | Multifunctional alkylating agent. Induces chromosomal aberrations and sister chromatid exchanges.                                                                                          |
| Etoposide           | Yes        | Inhibits topoisomerase II without intercalating into DNA. Induces single and double strand DNA breaks and DNA protein cross-links. Cell cycle block (G2/M), apoptosis.                             |
| Daunorubicin        | Yes        | Inhibits topoisomerases I and II. Inhibits RNA and DNA syntheses. Induces single strand DNA breaks. Cell cycle arrest (G2/M).                                                           |
| MNNG                | Yes        | Alkylating agent adding methyl groups to cellular macromolecules including DNA.                                                                                                          |
| Actinomycin D       | Yes        | Inhibits topoisomerase II with intercalating into DNA. Inhibits DNA and RNA syntheses. Blocks transcription by inhibiting RNA polymerase and facilitating the formation of an alternating DNA structure. Induces DNA breaks. |
| Streptonigrin       | Yes        | Inhibits topoisomerase II without DNA intercalation. Inhibits DNA and RNA syntheses. Induces DNA cleavage (metal ions are required). Induces production of reactive oxygen species (ROS). Radiomimetic drug. |
| Mitoxantrone        | Yes        | Inhibits topoisomerase II.                                                                                                                                                                |
| Doxorubicin         | Yes        | Inhibits topoisomerase II. Binds nucleic acids (DNA intercalator) resulting in inhibition of nucleic acid synthesis. Induces apoptosis. Reduction of doxorubicin results in the formation of superoxide anion radicals and, later, other ROS, which can lead to DNA damage. |
| Taxol (Paclitaxel)  | No         | A microtubular-interfering agent enhancing rate and yield of microtubular assembly and preventing depolymerization resulting in disruption of normal microtubular cytoskeleton. Microtubular disarray and cell cycle arrest (G2/M). |
| Tamoxifen           | No         | Anti-estrogen agent. Inhibits Protein kinase C by modifying its catalytic domain.                                                                                                          |
| Thapsigargin        | No         | Irreversibly binds and inhibits the ER Ca^{2+} pump. ER stress.                                                                                                                         |
| Tunicamycin         | No         | Inhibits protein N-glycosylation resulting in overload of the ER with misfolded proteins. ER stress.                                                                                       |
Figure 1. Potapova et al.
Figure 2. Potapova et al.
Figure 3. Potapova et al.
Figure 4. Potapova et al.
Figure 6. Potapova et.al.
