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Biological Responses of Glioma Cell to Chemotherapeutic Agents

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1. Introduction

Because gliomas are not curable surgically, development of effective adjuvant therapies is warranted. A chemotherapeutic agent temozolimide (TMZ) has been widely used not only because it is well tolerated and easily administrated orally but because various clinical trials had revealed that high grade gliomas could show objective response or stable disease to this compound (Stupp et al., 2005). The action of TMZ had been extensively studied primarily in leukemia and lymphoma cells. TMZ spontaneously decomposes in aqueous solution to form the cytotoxic methylating agent, and the cytotoxicity of TMZ appears to be mediated mainly through adduction of a methyl group to O^6 position of guanine (G) in genomic DNA. The methyl group can be removed from O^6-methylguanine by O^6-methylguanine-DNA methyltransferase (MGMT). If MGMT is deficient in the cell, however, O^6-methylguanine is not repaired, and incorporation of a thymine (T) rather than a cytosine opposite the O^6-methylguanine during the next cycle of DNA replication leads to the formation of GT mismatches in DNA. This triggers the DNA mismatch repair (MMR) system which removes the T, only to have the T reinserted during repair synthesis. Futile cycles of MMR triggered by GT mismatches can lead to a variety of outcomes in TMZ-treated cells (Figure 1).

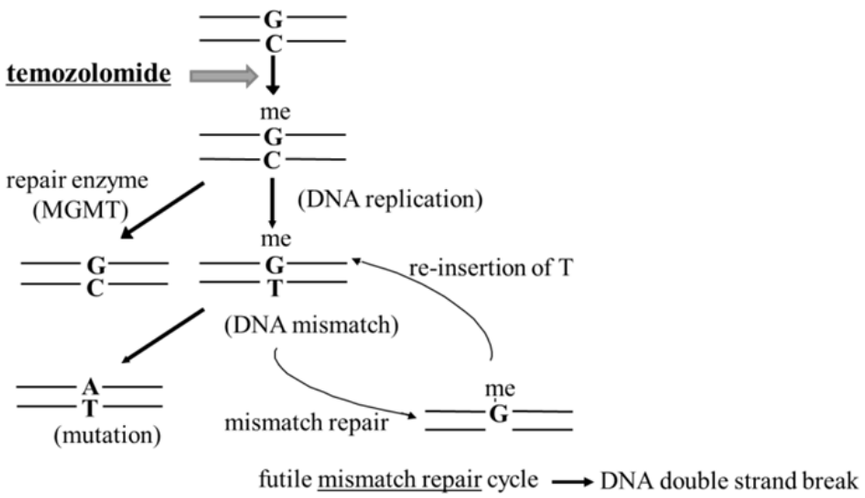


Fig. 1. Mechanism of temozolomide-induced DNA damage creation

Since MGMT has been considered as a key factor in the resistance of gliomas to TMZ, several clinical trials have been conducted. However, TMZ treatment in combination with MGMT-depleting compound did not show remarkable therapeutic affect for malignant gliomas, and recent clinical studies suggest that MGMT is just a prognostic marker for malignant gliomas treated with genotoxic agents including radiation and chemotherapy, and that MGMT is not the only factor that induces TMZ-resistance, and that further biological investigation on glioma cells is needed. In this chapter we review our studies on glioma biology in regard its cellular responses to DNA-damaging compounds, especially TMZ, which could provide a clue to develop safe and effective methods to potentiate anti-tumor activity of the drug.

2. Cell cycle arrest of glioma cells in response to temozolomide

Most of DNA-damaging chemotherapeutic agents induce cell cycle arrest, and so does TMZ (Hirose et al., 2001a). When MGMT-negative and p53 wildtype U87MG human glioblastoma cells were treated with TMZ at the concentration based on the published data of the plasma concentration of the drug in patients involved in its clinical trials (100 microM for 3 hours), FACS analysis revealed no significant difference in the percentage of cells in each phase of the cell cycle between untreated U87MG cells and the cells harvested at 1 day after TMZ treatment. However, cells began to accumulate at the G2/M boundary (4n DNA content) 2 days after TMZ treatment. This G2/M arrest (here defined as a greater percentage of cells in G2/M than G1) was sustained for at least 10 days after TMZ treatment, and was associated with the gradual appearance of hyperploid (>4n DNA content) cells and the gradual loss of cells with 2n DNA content (G1 cells). Although TMZ-treated cells underwent cell cycle arrest, the sub-G1 population, which represents apoptotic cells, was small and did not significantly increase throughout the 10 days following TMZ treatment. Consistent with the G2/M arrest data, p53 and $p21^{Waf1/Cip1}$ levels were increased approximately 2-4 fold at 2 days after TMZ treatment with the accumulation persisting at least 10 days after the treatment. On the other hand, **g**enetically modified U87MG-E6 cells which have low levels of p53 because of transfection of human papilloma viral oncoprotein E6 mediating ubiquitination and destruction of p53 showed shorter G2/M after TMZ treatment. That is, the proportion of U87MG cells at G2/M began decreasing by 3 days after TMZ treatment and was considerably lessened by 10 days after treatment. The reduction in G2/M arrest in these cells was also associated with a gradual increase in cells with a sub-G1 (less than 2n). Because U87MG and U87MG-E6 cells share a common genetic background except for expression of E6, comparison of the responses of these cells provides more useful information than the comparison of responses of cells with completely different genetic backgrounds. These results support the idea that p53 (and $p21^{Waf1/Cip1}$), while not important for initiation of TMZ-induced G2/M arrest, do play a role in sustaining the arrest (Figure 2).
Colony formation efficiency assay revealed that U87MG cells showed a dose-dependent decrease in clonogenicity, and, on the other hand, U87MG-E6 cells formed far less colonies. As well as being associated with the duration of TMZ-induced arrest in glioma cells, the p53 status of cells was also associated with the ultimate fate of the cells. p53-wildtype cells underwent a prolonged G2/M arrest which left the majority of cells viable yet non-proliferative showing the features of ascent cells. In contrast, p53-deficient cells underwent a more transient arrest, and lost viability in a manner consistent with mitotic catastrophy. Therefore while both p53-wt and p53-deficient cells became non-proliferative in response to

TMZ, the means by which this was accomplished differed in a manner consistent with p53 status.

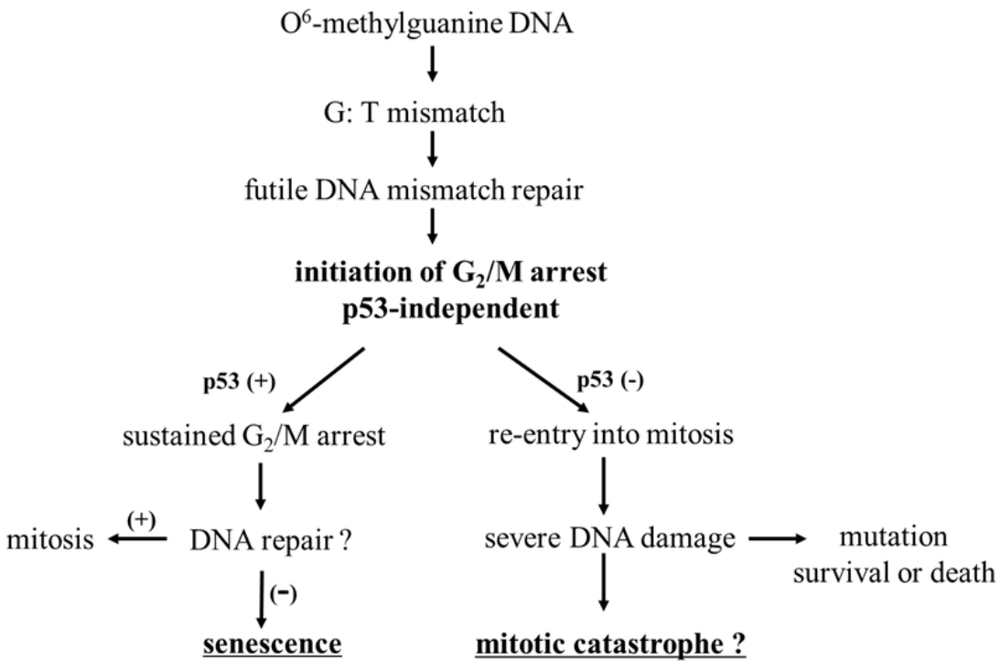


Fig. 2. TMZ-induced cell cycle arrest and the fate of the cells.

3. DNA checkpoint

While both p53-wt and p53-deficient cells initiate G2/M arrest and become non-proliferative in response to TMZ, p53-wt cells which undergo prolonged G2/M arrest are less sensitive than p53-deficient cells to the cytotoxic actions of TMZ. One possible explanation for this effect is that the prolonged G2/M arrest noted in p53-wt cells allows more time for reversal of the cytotoxic effects of the drug prior to entry into mitosis and death by mitotic catastrophe. G2/M arrest in response to TMZ may therefore represent a defense mechanism against the cytotoxic actions of TMZ.

While the linkage between TMZ-induced DNA damage and G2/M arrest has not been fully explored, the linkage between irradiation-induced DNA damage and G2/M arrest has been shown to involve a pathway controlling the cyclin-dependent kinase cdc2. Various types of DNA damage activate Chk1 kinase which phophorylates cdc25C phosphatase at serine-216 which enhances the binding of 14-3-3 proteins and the export of the cdc25C/14-3-3 complex to the cytoplasm. The cytoplasmic sequestration of phosphorylated cdc25C in turn eliminates the potential cdc25C-mediated dephosphorylation of cdc2. Cdc2 therefore remains bound to cyclin B in an inactive, phosphorylated state. The end result of DNA damage-induced Chk1 activation therefore is the phosphorylation of cdc2, and the arrest of cells with damaged DNA at the G2/M boundary. The ability of TMZ to induce DNA damage suggested that, like other DNA damaging agents, TMZ might initiate G2/M arrest via a Chk1-dependent pathway.

If Chk1 activation is critical in activation of G2/M arrest in TMZ-treated cells, and if G2/M arrest provides the opportunity for cells to avoid TMZ-induced cytotoxicity, inhibition of cdc2-dependent G2 arrest should sensitize cells to TMZ. A variety of small molecule inhibitors have recently been developed, and we analyzed the effect of UCN-01, a staurosporin derivative Chk1 inhibitor, as a pharmacologic tool to assess the linkage between TMZ exposure and G2/M arrest, to determine if G2/M arrest protects cells from TMZ-induced cytotoxicity, and to determine if Chk1 inhibitors might represent a way to sensitize cells to TMZ (Hirose et al., 2001b).

To better define how TMZ induces G2/M arrest, we analyzed alterations in levels of G2-checkpoint-associated proteins in TMZ-treated cells. The protein levels of Chk1 increased in a transient manner, rising at 1-2 days after TMZ exposure and returning to sub-control levels by 10 days after TMZ exposure. While total levels of cdc2 were unchanged or only slightly increased after TMZ, levels of phosphorylated cdc2 were transiently increased in both U87MG and U87MG-E6 cells in a timeframe and manner similar to that noted for induction of Chk1.

To more directly test the association between TMZ and alterations in G2 checkpoint proteins, U87MG and U87MG-E6 cells were treated with TMZ, and then exposed to UCN-01 for 3 days immediately following TMZ removal. Immunoblot analyses revealed that UCN-01 reduced the level of phosphorylated cdc2 in the cells and significantly inhibited TMZ-induced G2/M arrest of both U87MG and U87MG-E6 cells Furthermore, in agreement with the study described above, UCN-01 reduced TMZ-induced senescence-associated beta-galactosidase activity and enhances mitotic catastrophe. UCN-01 increases TMZ-induced cytotoxicity in both p53-wt and p53-deficient glioma cells (Figure 3).

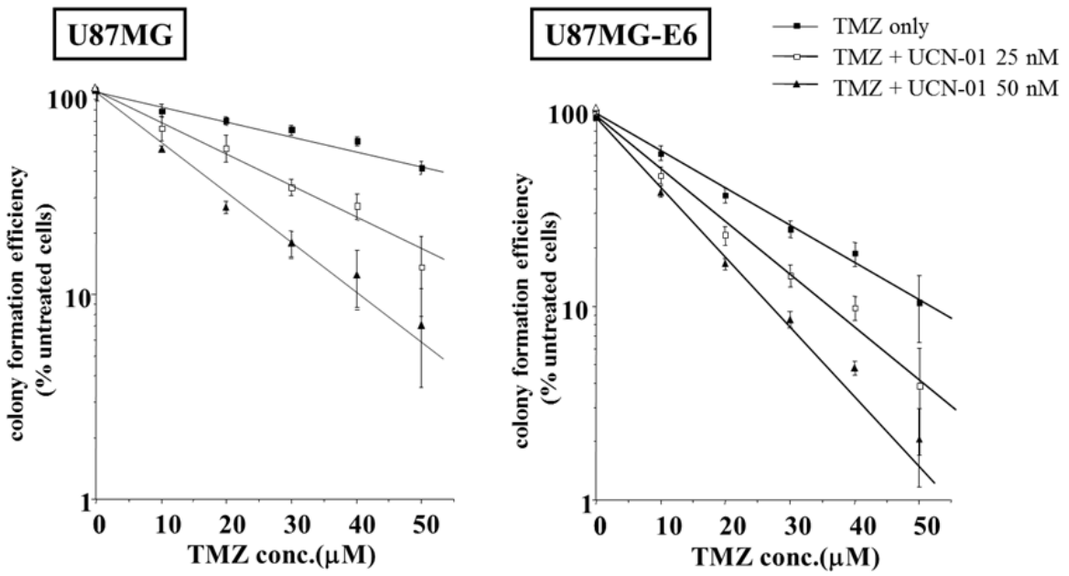


Fig. 3. p53-independent sensitization of glioma cells to TMZ by Chk1 inhibitor UCN-01.

Having established an association between TMZ-exposure, Chk1 activation, and TMZ-induced G2/M arrest, we examined the hypothesis that G2/M arrest is a protective response of cells to TMZ-induced cytotoxicity, and that elimination of the G2 checkpoint might sensitize cells (and in particular p53-wt cells) to TMZ. In p53 wt cells, which undergo

a p53-associated prolonged G2/M arrest and senescence in response to TMZ, UCN-01 post-treatment greatly reduced the extent of G2/M arrest, reduced the percentage of cells undergoing TMZ-induced senescence, and increased the percentage of cells undergoing mitotic catastrophe. These results clearly suggested that in p53-proficient glioma cells, the G2 checkpoint serves a protective function, and that elimination of the checkpoint is associated with an increase in the number of cells that die by pre-mature entry into mitosis. UCN-01, however, not only sensitized p53-wt cells but also p53-deficient cells which underwent only a transient G2/M arrest in response to TMZ. This sensitization did not involve changes in TMZ-induced senescence (which was minimal in these cells) but rather was associated exclusively with increases in the number of cells undergoing mitotic catastrophe (Figure 4).

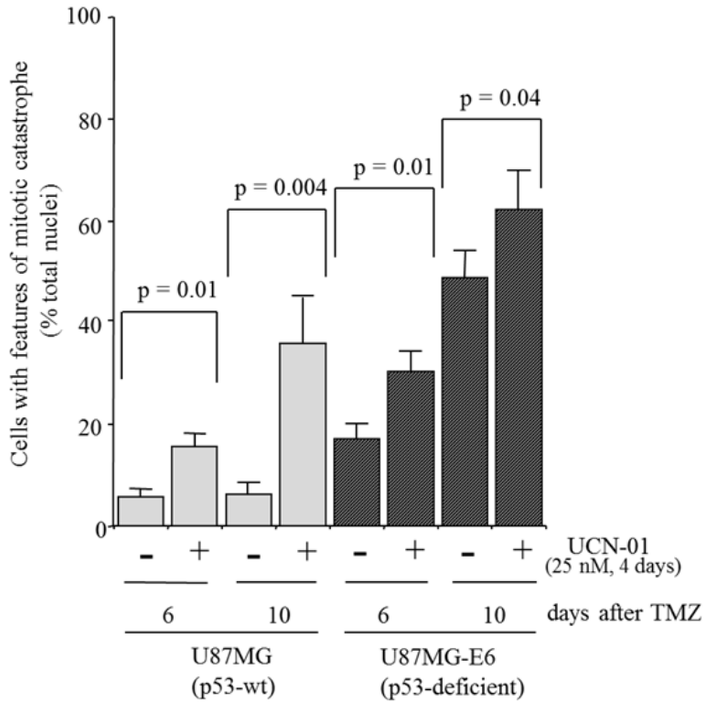


Fig. 4. Enhancement of TMZ-induced mitotic catastrophe by UCN-01.

While increased levels of MGMT and loss of MMR capacity can both confer TMZ resistance, very few gliomas over-express MGMT or are MMR deficient. It appears likely, therefore that at least some of the resistance of gliomas to TMZ involves events downstream of futile MMR activation. The ability of UCN-01 to prevent downstream events which may contribute to TMZ resistance may therefore prove useful in the treatment of TMZ-resistant as well as TMZ-sensitive tumors. As approximately two-thirds of gliomas have defects in the p53 pathway, the ability of UCN-01 to sensitize cells regardless of p53 status also increases the range of tumors for which this approach might be effective. While issues relating to duration of exposure, drug sequencing, and the events that link MMR to Chk1 activation remain to be examined, it has been suggested that the combinations of TMZ with G2 checkpoint inhibitors such as UCN-01 might be useful additions to existing therapies for brain tumors.

4. Stress-activated kinases

Stress-activated kinases (SAPKs) could be targeted in an effort to enhance the effect of chemotherapeutic agents because the tumor cells, which grow under various types of cellular stress including hypoxia and genetic instability, might survive in severe environment by modifying on stress-inducing events. We here discuss about two SAPKs, p38MAPK and c-Jun-N-terminal kinase (JNK).

4.1 p38 MAPK

While the cellular response to methylating agent exposure appeared highly dependent on DNA repair processes influenced by the G2 cell cycle checkpoint, the purpose of the cell cycle arrest remains unclear, although it has long been suggested that cell cycle arrest provides cells that have incurred DNA damage time in which to reverse the damage. Despite this suggestion has not been directly tested, it was clear that the prolonged G2 arrest noted in glioma cells exposed to cytotoxic methylating agents alters their response.

It has been appreciated for some time that cells lacking a functional MMR system undergo neither G2 arrest nor cytotoxicity in response to methylating agents, clearly suggesting a link between MMR and G2 arrest.

A potential signaling pathway that might help connect activation of the MMR system to G2 arrest is that controlled by the p38 MAPK family. The p38 family of stress kinases contains four members: α, β, γ, and δ. These MAP kinases are activated by the upstream kinases MKK3 and/or MKK6 in response to diverse stimuli including environmental stress and growth factors. p38 kinases in turn activate a variety of downstream targets including MAPKAP2, the C/EBP family of transcription factors, and various other transcription factors including p53. In this manner p38 is believed to play an important, although not well defined, role in co-ordinating a variety of cellular events including cell growth, cell differentiation, and cell death. At least two recent studies have suggested that the ability of the p38 pathway to co-ordinate cell growth and cell death might also extend to actions on cell cycle progression.

To begin to address the possible role of the p38 pathway in the response of cells to methylating agents, we exposed U87MG cells to TMZ, and the various p38 isoforms were then immunoprecipitated from the nuclear fractions, and equal amounts of the immunoprecipitated p38 isoforms from each time point were incubated with the p38 substrate ATF2 in the presence of ATP. TMZ exposure stimulated p38α kinase activity and p38β kinase activity was also modestly stimulated while the activity of p38γ and δ were not significantly affected (Hirose et al, 2003).

To investigate linkage between MMR and p38 activation, we first exposed MMR-deficient HCT116 human colorectal adenocarcinoma cells and paired MMR-proficient HCT116 cells containing a copy of *Mlh1*-containing chromosome 3 (HCT116 3-6 cells) to TMZ, after which the cells were collected and analyzed for levels of p38α phosphorylation/activation and extent of G2 arrest by Western blot and by FACS analysis, respectively. Neither HCT116 nor HCT116 3-6 cells exhibited p38 induction or G2 arrest in the first two days following TMZ exposure, consistent with previous studies showing that both these cell lines are MGMT-proficient and presumably repair TMZ-induced O^6-methylguanine before these lesions mispair with thymine and trigger downstream consequences. Following exposure to the highly specific MGMT depleting agent O^6-benzylguanine prior to and after TMZ exposure, however, the MMR-proficient HCT116 3-6 cells exhibited TMZ-induced p38α

activation in a manner similar to that noted in U87MG glioma cells, and underwent G2 arrest. O^6-benzylguanine-exposed, TMZ-treated MMR-deficient HCT116 cells, however, exhibited neither p38 activation nor G2 arrest. These results suggested in a preliminary manner that p38 activation in response to TMZ is dependent on O^6-methylguanine lesions and on the recognition and/or processing of these lesions by the DNA MMR system.

While studies in HCT cells suggested an association between the DNA MMR system and TMZ-induced G2 arrest, the MMR-corrected HCT116 3-6 cell line used was derived from a clone that contains a large portion of human chromosome three, and which therefore could differ from the MMR-proficient HCT116 cells in ways more dependent on clonal selection and multiple gene expression that on direct correction of the MMR defect. We therefore also examined TMZ-induced p38 activation and G2 arrest in MGMT-deficient, MMR-proficient human U87MG glioblastoma cells and in paired cells made MMR-deficient by expression of a retrovirally-encoded siRNA targeted to the MMR protein MLH1. Expression of the retrovirally encoded MLH1 siRNA blocked TMZ-induced G2 arrest such that cells expressing the MLH1 siRNA, but not cells expressing the blank vector, avoided TMZ-induced G2 arrest in a manner identical to that noted in HCT116 cells in which MLH1 was eliminated by mutation. More importantly, siRNA-mediated reduction of MLH1 levels also blocked the ability of the cells to activate the p38 pathway. These results, in connection with those derived from the studies with HCT cells, suggested that p38 activation was a common response of human cells to the methylating agent TMZ, that this activation is dependent on a functional MMR system, and that p38 activation is associated with methylating agent-induced G2 arrest.

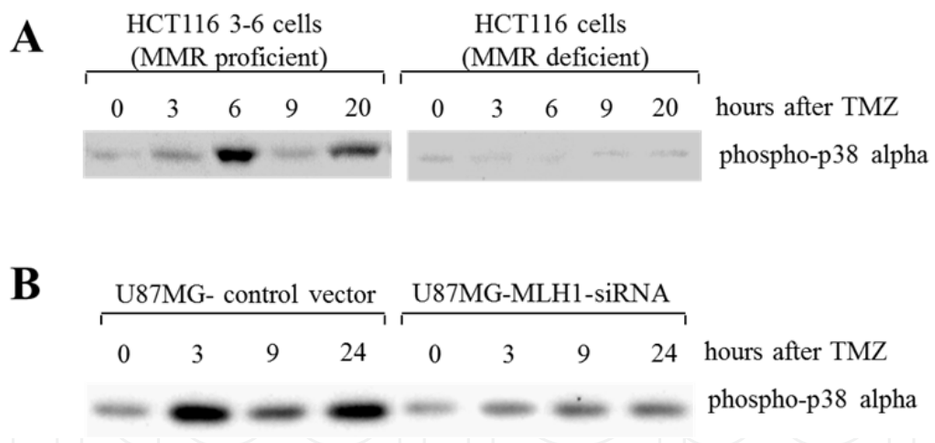


Fig. 5. TMZ-induced p38 activation in MMR-deficient colon carcinoma cells (A) and MMR-silenced glioma cells (B).

Having demonstrated the linkage between DNA MMR and p38 activation, we wished to more clearly define the potential linkage between p38 activation and G2 arrest. To do so we used both pharmacologic and genetic inhibitors of p38α, and monitored the effects of these inhibitors on G2 checkpoint proteins and on TMZ-induce G2 arrest. For pharmacologic inhibition studies, MGMT-deficient, MMR-proficient U87MG cells were exposed to the p38α/β selective inhibitors SB203580 or SB202190 prior to and following TMZ exposure, after which effects of p38 inhibition on TMZ-induced p38 activation, activation of the G2 checkpoint pathway, and activation of the G2 checkpoint itself were examined. Both

compounds not only blocked TMZ-induced p38 activation but also dramatically inhibited the ability of U87 cells to undergo G2 arrest two days following TMZ exposure. Exposure of cells to SB203580 prior to and following TMZ exposure blocked the inactivation of both cdc25C and cdc2, consistent with the inability of SB compound-treated cells to undergo G2 arrest following TMZ exposure. SB203580 exposure, however, had no significant effect on TMZ-induced Chk1 or Chk2 activation. These results suggest that the p38 pathway, and p38α/β specifically, are linked not only to MMR but also to activation of the G2 checkpoint through Chk1/2-independent actions on cdc25C and/or cdc2.

We also employed genetic means to selectively inhibit p38α and to assess the consequences of p38α inhibition on the G2 arrest pathway. To do so, MGMT-deficient, MMR-proficient U87MG cells were transfected with a pool of siRNA targeting p38α, after which the cells were exposed to TMZ. Transfection of U87MG cells with p38α siRNA reduced basal levels of p38α, and blocked the TMZ-induced ability of immunoprecipitated p38α to phosphorylate the p38 substrate MAPKAP2 *in vitro*. Selective genetic inhibition of p38α blocked TMZ-induced G2 arrest in U87MG cells in manner identical to that mediated by pharmacologic inhibitors of p38α/β. Furthermore, as was noted in studies using pharmacological inhibitors, p38α siRNA blocked the phosphorylation/inactivation of both cdc25C and cdc2 without affecting TMZ-induced Chk1 or Chk2 activation (Figure 6). These results clearly show that p38α is activated by the DNA MMR system, and that this activation is in turn linked by Chk1- and Chk2-independent means to inactivation of cdc2, cdc25C, and ultimately to G2 arrest.

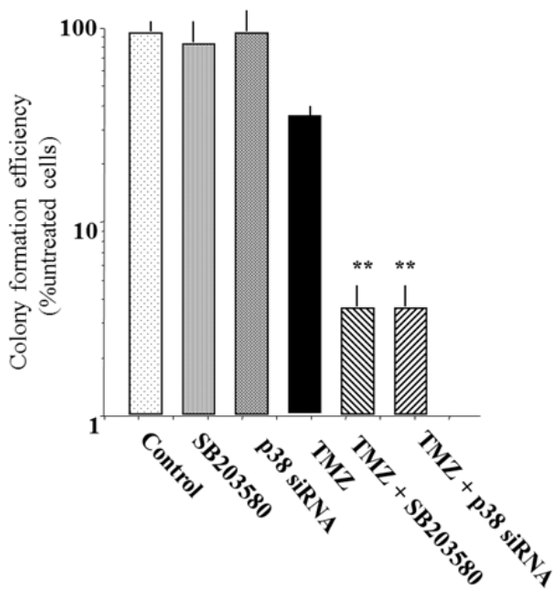


Fig. 6. p38 inhibition potentiated TMZ-induced cytotoxicity

Although pharmacologic or genetic inhibition of p38α did not alter clonagenicity, extent of senescence-associated beta-galactosidase expression, or extent of mitotic catastrophe, (data not shown), it did enhance the sensitivity of U87MG cells to TMZ-induced cytotoxicity, reducing the colony forming ability of these cells. p38 inhibition increased the percentage of TMZ-treated cells that died by mitotic catastrophe, consistent with the

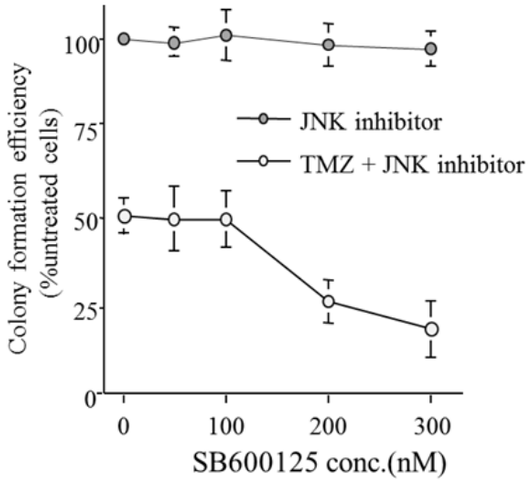
idea that cells incapable of undergoing G2 arrest enter mitosis with damaged DNA and die by mitotic catastrophe. These results suggest that p38α not only links MMR to the G2 checkpoint, but also influences the response of cells to cytotoxic methylating agents. It would be reasonable to suspect that tumors capable of activating the G2 checkpoint via the p38α pathway would be less sensitive than those incapable of doing so. Conversely, because inhibition of p38α sensitizes cells to TMZ, p38α might be a reasonable therapeutic target.

4.2 c-Jun N-terminal kinase

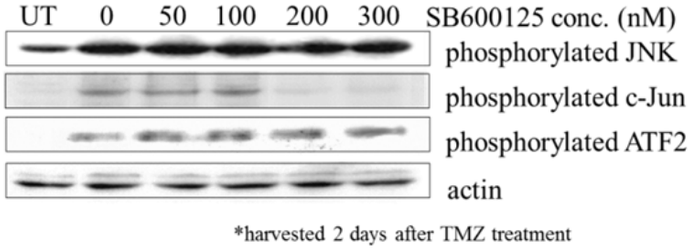
Since p38, a major component of mitogen-activated protein kinases (MAPKs), had been shown to be involved in TMZ-induced cellular responses, we were interested other MAPKS in terms they could be target for chemosensitization. MAPKs are components of a complex intracellular signaling network that regulates gene expression in response to extracellular stimuli, in turn regulating cell proliferation, differentiation, and cell death. The MAPK family includes components of three major pathways in humans: p38 kinase, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase. In the JNK pathway, specific stimuli activate various kinases leading to activation of JNK. Once activated, JNK is translocated to the nucleus, where it phosphorylates and activates transcriptional factors such as components of activator protein, including c-Jun, JunB, and JunD, as well as other factors including ATF-2 and STAT-3. As examples of the importance of JNK on cell signaling, JNK is associated with cell survival, oncogenesis, growth, differentiation, and cell death. The role of the JNK pathway in the mediation of cellular responses (including cellular transformation, cell growth, and cell death) to extracellular stimuli has been studied extensively. Interestingly, JNK can exert completely opposite effects depending on the cell type and stimuli. Since the question of whether JNK is associated with cell death or cell survival appeared to depend on the type of cellular stress, we investigated in the role of JNK in glioma cells treated with TMZ (Ohba et al., 2009).

We first investigated whether JNK was activated in response to TMZ and confirmed the activation of JNK in glioma cells treated with TMZ. Previous studies on gliomas had already shown that JNK was activated and played a pro-survival role in response to the DNA crosslinking agent CDDP, and that the inhibition of the JNK pathway sensitized glioma cells to CDDP. Sensitization to chemotherapeutic agents by the inhibition of the JNK pathway has also been reported either in other cancer cells although, on the other hand, the inhibition of JNK reportedly led to the suppression of chemotherapeutic agent-induced apoptosis in several studies. Therefore, we investigated the role of JNK in glioma cells treated with TMZ by utilizing the JNK inhibitor SP600125. As a result, we confirmed that SP600125 potentiated the TMZ-induced cytotoxicity in U87MG cells, and concluded that JNK activation played a cytoprotective role in glioma cells in response to TMZ-induced DNA damage (Figure 7A).

To clarify the mechanism responsible for the JNK inhibitor-induced potentiation of TMZ-induced cytotoxicity, the two main downstream proteins of JNK, c-Jun and ATF-2, were investigated, since these proteins are believed to be associated with chemoresistance. In our study, because SP600125 inhibited the phosphorylation of c-Jun but not of ATF-2 at the low concentration at which SP600125 induced chemosensitization to TMZ, c-Jun-related responses were considered to be more important in the JNK-mediated survival of glioma cells treated with TMZ than ATF-2-related pathways (Figure 7B).



(A)



(B)

Fig. 7. Effect of JNK inhibitor SB600125 on TMZ-induced cytotoxicity (A) and phosphorylation of associated proteins (B).

As noted above, the inhibition of p38 increased the sensitivity of glioma cells to TMZ in association with the abrogation of G2 arrest, however, in contrast, JNK inhibitor did not affect the cell cycle distribution of TMZ-treated cells nor changed the protein level of phosphorylated cdc2. Thus, the SP600125-induced chemosensitization to TMZ in glioma cells was probably not a consequence of the abrogation of cell cycle arrest. Rather, JNK inhibition increased the percentage of senescence-like cells in U87MG cells and of mitotic catastrophe cells in U87MG-E6 cells after treatment with TMZ. These results suggest that the enhancement of TMZ toxicity by a JNK inhibitor in glioma cells was induced by the potentiation of cell death pathways induced by TMZ alone. These data might be in agreement with previous studies on gliomas which suggested involvement of the JNK pathway in DNA repair.. c-Jun-related responses could be key events in the JNK-mediated cytoprotection of glioma cells treated with TMZ, and further investigations of the involvement of c-Jun in the survival machinery of cells with DNA damage might promote the development of useful chemotherapeutic strategies against malignant gliomas.

5. Survival-promoting protein Akt

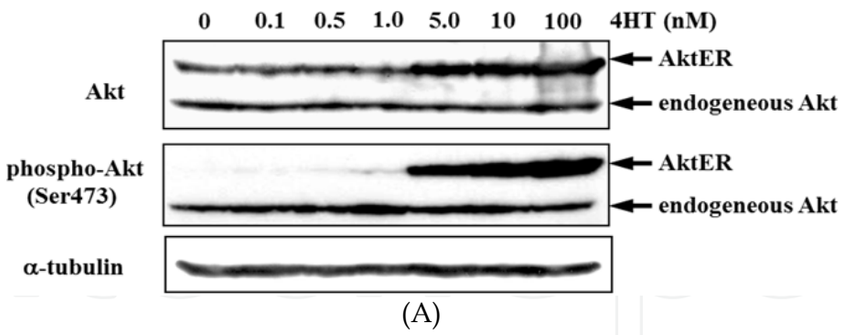
Survival promoting protein such as Akt, which is frequently activated in malignant gliomas, could be a target to enhance the effect of chemotherapeutic agents.

Whereas an extensive network of proteins are required to work together to initiate G2 arrest in response to DNA damage, a number of additional proteins have been reported to alter activation and maintenance of the G2 checkpoint. One of the most interesting of these is Akt, a member of the phosphatidylinositol-3 kinase family that is recruited to the cell membrane and activated in response to the generation of phospholipids by a variety of signaling pathways. Activated Akt in turn signals to a variety of key downstream molecules including mammalian target of rapamycin (mTOR), glycogen synthetase kinase 3, and S6 kinase, the sum of which is to suppress cell death and to promote cell survival. In addition to effects on apoptosis and cell metabolism, Akt activation has been reported to suppress activation of the G2 checkpoint in human colon carcinoma cells exposed to radiation. Whereas the mechanism by which Akt suppresses G2 arrest has not been defined, the observation that Akt influences the G2 checkpoint is of particular importance to the therapeutic application of TMZ whose cytotoxicity is influenced by the G2 checkpoint and which are frequently used in the treatment of PTEN-deficient, Akt overexpressing gliomas. Furthermore, because exposure of glioma cells to TMZ induces a senescence-like phenomenon and mitotic catastrophe, and because bypass of TMZ–induced G2 arrest by Chk1 inhibitors enhances, rather than suppresses, glioma cell death as described above, the consequences of potential Akt-mediated bypass of methylating agent–induced G2 arrest on cellular outcome remained uncertain.

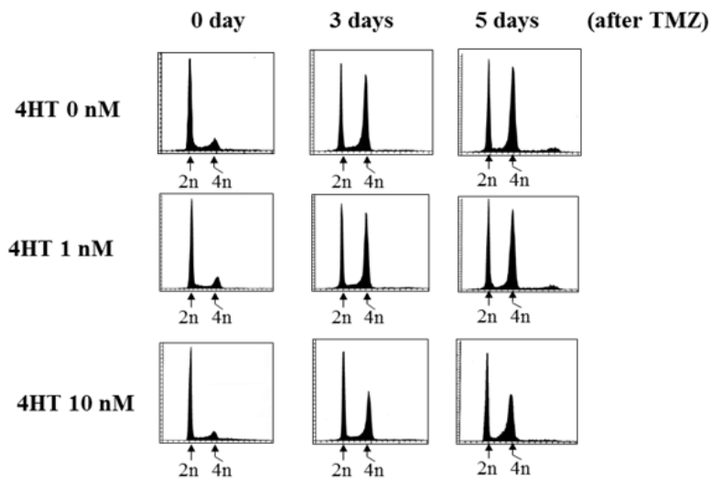
To investigate the effect of Akt activation in biological responses of glioma cells to DNA-methylating compound, we created U87MD-derived cells of which Akt activity could be exogeneously controlled. U87MG human glioma cells were infected with a retroviral construct encoding a modified Akt (AktERM+) protein which, by virtue of deletion of the parathyroid hormone domain, fusion to a c-Src myristoylation signal, and fusion to a modified form of the mouse ER hormone binding domain, has been shown rapidly activated in response to 4-hydroxytamoxifen. The levels of AktER rose in response to exposures of 4-hydroxytamoxifen (Figure 8A).

Having created cells with inducible levels of activated Akt, we addressed the consequences of Akt pathway activation on TMZ-induced G2 arrest. In the absence of 4-hydroxytamoxifen, TMZ-treated U87MG-AktERM+ cells showed Cdc2 (Tyr15) phosphorylation/ inactivation, and G2 arrest 3 to 5 days following TMZ exposure. However, U87MG cells expressing the AktERM+ construct exhibited significant inhibition of TMZ-induced phosphorylation/ inactivation of Cdc2, and TMZ-induced G2 arrest (Figure 8B).

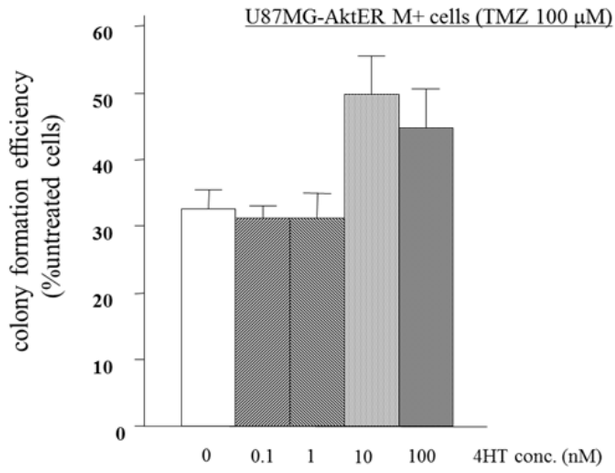
As described above, inhibition of TMZ-induced G2 arrest by Chk1 inhibitor forced cells into mitosis and enhances cell death by mitotic catastrophe in both p53-proficient and p53-deficient glioma cells. We therefore questioned whether bypass of TMZ-induced G2 arrest by Akt, a protein known to suppress apoptosis in response to radiation-induced DNA damage, would sensitize cells to TMZ or instead protect cells by suppressing pathways linked to senescence and/or mitotic catastrophe. As a result, AktERM+ activation protected rather than sensitized the p53-proficient U87MG cells to TMZ-induced cytotoxicity (Figure 8C). The cytoprotective effects of Akt overexpression were associated with a reduction in the percentage of cells expressing senescence-associated beta-galactosidase activity following TMZ exposure, suggesting that Akt overexpression not only reduced the percentage of cells undergoing TMZ-induced G2 arrest but also reduced the ability of p53-positive glioma cells to undergo a senescence-like phenomenon in response to TMZ. Furthermore, AktERM+



(A)



(B)



(C)

Fig. 8. Akt over-activation in U87MG-AktERM+ cells (A) and its effect on TMZ-induced cell cycle arrest (B) and cytotoxicity (C)

overexpression in U87MG-E6 cells suppressed not only TMZ-induced G2 arrest and loss of clonogenicity but also the percentage of cells undergoing death by mitotic catastrophe following TMZ exposure. These results suggest that Akt overexpression, whereas suppressing TMZ-induced G2 arrest, also protects cells from loss of clonogenicity caused by induction of senescence and mitotic catastrophe.

In summary, the results showed that Akt activation suppresses the G2 checkpoint by selectively altering activation of the DNA damage signal transducer Chk2 and the downstream effectors of the G2 checkpoint. The overriding effect of Akt activation, however, is suppression of TMZ-induced senescence and mitotic catastrophe in cells that avoid G2 arrest. The Akt pathway may therefore contribute to TMZ resistance in the clinical setting.

It is interesting to note that most high-grade human gliomas have high levels of Akt activation, which are believed to be a consequence of PTEN deletion. Given the role Akt plays in moving cells through the G2 checkpoint and in suppressing TMZ-induced cytotoxicity, it seems likely that an analysis of Akt pathway activation in gliomas before therapy may help identify those individuals for whom TMZ-based therapies are most likely to succeed. Similarly, strategies combining TMZ with inhibitors of the Akt pathway may enhance the likelihood of success. Because Akt overexpression has also been reported to increase the mutagenicity of agents that induce G2 arrest, presumably by promoting cell survival in the absence of genuine DNA repair, strategies designed to suppress Akt may contribute not only to improved tumor cell kill but also to suppression of unwanted mutagenic effects which might otherwise contribute to secondary malignancies.

6. Molecular chaperone 90kD heat shock protein

As reviewed above, several ways to potentiate the cytotoxicity of TMZ have been reported, and thus many pathways can be targeted in an effort to sensitize tumor cells to chemotherapeutic agents. A molecular chaperone, 90kD heat shock protein (hsp90) has recently attracted attention as a sensitizing agent because it is expressed at 2–10-fold higher levels in tumor tissue than in normal tissue, and is associated with many proteins (termed client proteins) involved in cell cycle regulation, cell survival and oncogenesis. Taken together, these studies indicated that many hsp90 client proteins are involved in cytoprotective mechanisms against cellular stressors such as DNA damage, suggesting that hsp90 might be important in the survival of tumor cells after exposure to DNA-damaging chemotherapeutic agents. Therefore we hypothesized that hsp90 inhibitors might act as antitumor agents against gliomas and might potentiate the cytotoxicity of DNA-damaging agents.

To investigate this hypothesis, we used 17-AAG, a geldanamycin derivative, as a pharmacological inhibitor of hsp90 and examined whether an hsp90-targeted strategy could be useful for chemosensitizing glioma cells to the DNA-damaging agents BCNU, cisplatin, and TMZ (Ohba et al., 2010).

The clonogenicity of cells treated with cisplatin, BCNU, or TMZ was depressed with 17-AAG. This 17-AAG–induced potentiation of the effects of these chemotherapeutic agents was recognized at a lower concentration than that needed to induce cytotoxicity with 17-AAG alone, and was more remarkable in the cisplatin- and BCNU-treated cells than in the

TMZ-treated cells. Isobologram analyses revealed that the interactions between 17-AAG and cisplatin or BCNU were synergistic, whereas the interaction between 17-AAG and TMZ was no more than additive (Figure 9).

The FACS analyses revealed that the population of cells with a sub-G1 DNA content was increased by combined treatment with 17-AAG and either cisplatin or BCNU; furthermore, the combined treatment remarkably increased the number of annexin V–positive and PI-negative cells. These results demonstrated that the 17-AAG– induced enhancement of the DNA crosslinking agents–induced cytotoxicity was either associated in part or entirely with an increase in apoptotic cell death.

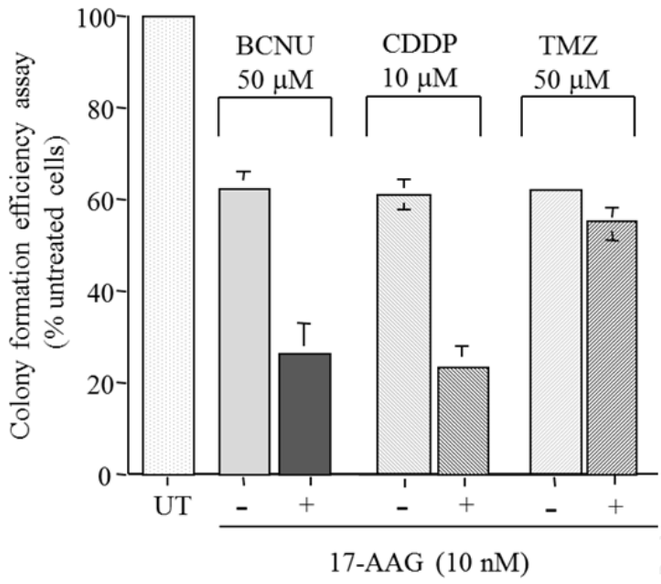


Fig. 9. Effect of Hsp90 inhibitor 17-AAG on cytotoxicity induced by various DNA damaging agents.

A reasonable concern is that hsp90 inhibitors may act not only on tumor cells, but also on normal cells; however, the authors of previous studies have shown that hsp90 is expressed at 2–10-fold higher levels in tumor than in normal tissue, and that hsp90 derived from

tumor cells has a 100-fold higher binding affinity for the hsp90 inhibitor 17-AAG than does hsp90 derived from normal cells. These data suggest that the effect of hsp90 inhibitors on normal cells may be much smaller than that on tumor cells and that an hsp90 inhibitor might therefore be useful for selectively killing tumor cells.
The mechanism of the 17-AAG–induced enhancement of the cytotoxicity of DNA-crosslinking agents has not yet been completely elucidated, and suppression of other survival-promoting factor(s) could be involved in enhancement of DNA-damaging agents, however.

7. Conclusion

We reviewed our studies focusing chemosensitization of gliomas. We propose that combination of conventional chemotherapy using DNA-damaging agents and molecular targeted therapy could be a potentially useful new antiglioma therapeutic strategy. However, enhancement of the effect of chemotherapeutic agents clearly depends on the mechanism by these compounds exhibit cytotoxicity. Therefore the development of a safe and effective therapeutic regimen will require further investigation

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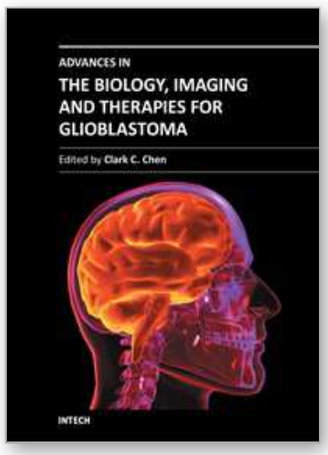
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This book is intended for physicians and scientists with interest in glioblastoma biology, imaging and therapy. Select topics in DNA repair are presented here to demonstrate novel paradigms as they relate to therapeutic strategies. The book should serve as a supplementary text in courses and seminars as well as a general reference.

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