Contribution of ATM and ATR to the resistance of glioblastoma and malignant melanoma cells to the methylating anticancer drug temozolomide

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Summary

The major cytotoxic DNA adduct induced by temozolomide and other methylating agents used in malignant glioma and metastasized melanoma therapy is O\textsuperscript{6}-methylguanine. This primary DNA damage is converted by mismatch repair into secondary lesions, which block replication and in turn induce DNA double-strand breaks that trigger the DNA damage response (DDR). Key upstream players in the DDR are the PI3 kinases ATM and ATR. Here we addressed the question of the importance of ATM and ATR in the cell death response following temozolomide. We show that a) ATM and ATR mutated cells are hypersensitive to temozolomide, b) O\textsuperscript{6}-methylguanine triggers ATM and ATR activation, c) knockdown of ATM and ATR enhances cell kill in glioblastoma and malignant melanoma cells with a stronger effect in ATR knockdown cells, d) ATR, but not ATM knockdown abolished phosphorylation of H2AX, CHK1 and CHK2 in glioma cells, and e) temozolomide-induced cell death was more prominently enhanced by pharmacological inhibition of CHK1 compared to CHK2. The data suggests that ATM and, even better, ATR inhibition is a useful strategy in sensitizing cancer cells to temozolomide and presumably also other anticancer drugs.
Introduction

Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are both serine/threonine protein kinases important for DNA damage processing. ATM mutated in the homozygous state gives rise to the chromosomal instability syndrome Ataxia telangiectasia (A-T, also known as Louis-Bar syndrome) (1). The patients exhibit a strong predisposition to cancer formation and are extremely sensitive to ionizing radiation (2). Homozygous mutation of ATR gives rise to the Seckel syndrome, which is not radiation sensitive, but phenotypically the patients exhibit other peculiarities such as dwarfism (3).

ATM is activated by DNA double-strand breaks (DSB). Active ATM phosphorylates several target proteins, which are involved in cell cycle control, DSB repair and the initiation of autophagy and apoptosis. Among them are histone 2AX (H2AX) (4) and CHK2 (5). The latter induces the so-called ATM-CHK2-pathway to block cell cycle progression. In contrast to ATM, ATR is activated by stalled replication forks (6). Similar to ATM, activated ATR kinase phosphorylates a variety of downstream targets including H2AX (7) and CHK1. CHK1 is then responsible for the ATR dependent cell cycle arrest (8). Functionally, the ATM-CHK2- and the ATR-CHK1-pathway are not strictly separated from each other. Thus, CHK1 phosphorylation in response to ionizing radiation is dependent on ATM (9) and the phosphorylation and consequential activation of ATM caused by UV light and stalled replication forks are dependent on ATR (10). Furthermore, CHK2 can be a substrate of ATR in response to ionizing or UV irradiation (11). Clearly there is either considerable crosstalk between the ATM-CHK2- and ATR-CHK1-pathways or the processing of the primary DNA lesions lead to secondary DNA structures that activate both.

The anticancer drug temozolomide (TMZ) and the model S₃,N1-methylating agent N-methyl-N' nitro-N-nitrosoguanidine (MNNG) methylate DNA at 13 different positions (12) forming the main product N7-methylguanine (N7MeG) and the minor adducts N3-methyladenine (N3MeA), N3-methylguanine (N3MeG) and O⁶-methylguanine (O⁶-MeG). From these adducts O⁶-MeG is clinically most important as it is the main mutagenic,
genotoxic and killing lesion (13). O\textsuperscript{6}-MeG, induced at less than 8% of total methylations by methylating agents, is repaired by O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT), which is then targeted for proteosomal degradation (14). Unrepaired O\textsuperscript{6}-MeG triggers apoptosis in a mismatch repair (MMR) (15) and DNA replication (16) dependent manner. As O\textsuperscript{6}-MeG is a cytotoxic lesion, the level of killing resistance is dependent on the amount of MGMT in the cell and the re-synthesize rate of the enzyme. The importance of MGMT in the protection against methylating chemotherapeutics is reflected by the fact that these drugs are used for the treatment of tumors, malignant glioma and metastatic melanoma, that express low amounts of MGMT (17).

ATM and ATR are activated in a MMR dependent manner (18). ATR was reported to be activated in the 2\textsuperscript{nd} cell cycle following methylation by methylating agents in synchronized cells (19). In addition, the phosphorylation of CHK1 and p53 upon methylating agent treatment was shown to be dependent on ATR and, along with CHK2, independent of ATM (18). Whether these dependencies were triggered by O\textsuperscript{6}-MeG adducts requires further studies. Both ATR and ATM can activate the G2/M cell cycle arrest following methylation (20, 21). Loss of functional ATM sensitizes mouse fibroblasts to O\textsuperscript{6}-MeG lesions (22). In addition, KU55933, a selective ATM inhibitor, sensitizes glioma cells to TMZ due to an increased accumulation of DSBs (23). Whether this was a result of O\textsuperscript{6}-MeG adducts in glioma cells was not addressed. These studies differ from earlier studies done on lymphoblastoid and fibroblast cell lines isolated from A-T patients where no increased sensitivity towards alkylating agents was observed (24, 25).

The present study was performed to determine the extent of protection that ATM and ATR exert in glioblastoma and malignant melanoma cells against cytotoxicity induced by the critical toxic lesion O\textsuperscript{6}-MeG. The results show that ATR plays a dominant role in causing drug resistance compared to ATM that contributes to a lesser extent. The data also show that ATR activates H2AX, CHK1 and CHK2 in response to O\textsuperscript{6}-MeG and that the inhibition of CHK1 or CHK2 sensitizes cells to this DNA lesion. As the results were obtained in
glioblastoma and malignant melanoma cells they will be of interest for these tumor types during therapy.

**Material and Methods**

**Cell lines and culture conditions**

All the cell lines used in this study are of human origin. Three SV40-immortalized fibroblast cell lines (GM637 [wt], ATs4 [ATM mt] and GM05849 [ATM mt]), two EBV-immortalized lymphoblastoid cell lines (AG09387 [wt] and DK0064 [ATR mt]), one melanoma cell line (D03) and one glioma cell line (LN229). The GM637 cells were described in (26), while the ATs4 cells were described in (27). The line GM05849 was from the Coriell Institute (Camden, New Jersey). The lymphoblastoid cell lines AG09387 and DK0064 were a gift from M. O'Driscoll (Brighton, UK). The melanoma cells D03, described in (28), were a gift from C.W. Schmidt (Queensland, Australia) and the glioblastoma cell line LN229, described in (29), was from M. Weller (Zürich, Switzerland). All the lines were carefully characterized in the laboratory they originated from, displayed the expected phenotype, but were not re-authenticated in our laboratory. All of them, except for LN229, were cultivated in RPMI containing 10 % FCS and penicillin/streptomycin (10 U/ml, 0.1 mg/ml). LN229 was cultured in DMEM containing 10 % FCS and penicillin/streptomycin (10 U/ml, 0.1 mg/ml). All cells were kept in a humid atmosphere with 5 % CO₂ at 37 °C. LN229-MGMT-12 is a cell line stably over-expressing MGMT, the generation of which has been described previously (30). LN229-MGMT-12 was maintained in medium containing 0.75 mg/ml G418. G418 was omitted from the medium during experiments. To maintain genome stability the cell cultures were frequently replaced by new stocks stored in liquid nitrogen. Doubling times for all cell lines except GM05849 and the MGMT transfectants are shown in supplemental Table 1.

**Drug treatment**
Cells were exposed to the genotoxic agents MNNG (Sigma-Aldrich, Taufkirchen, Germany) and TMZ (Schering-Plough, Kenilworth, New Jersey) by adding the drugs once to the medium. Unless otherwise stated, 1 h before the addition of MNNG or TMZ, 10 µM O6-benzylguanine (O6-BG, Sigma-Aldrich, Taufkirchen, Germany) was added to the medium to inactivate MGMT. In the experiment with LN229-MGMT-12 the O6-BG treatment was repeated 24 and 48 h after TMZ treatment (2.5 µM) in order to maintain long-term MGMT inactivation. The CHK1-inhibitor (UCN-01, Sigma-Aldrich, Taufkirchen, Germany) and the CHK2-inhibitor (CHK2-Inhibitor II, Sigma-Aldrich, Taufkirchen, Germany) were added to the medium once 24 h after treatment with TMZ to the final concentrations of 150 nM (UCN-01) and 10 µM (CHK2-Inhibitor II).

**Transient transfection of the tumor cells with siRNA targeting ATM- and ATR-mRNA**

Transient protein knockdown was accomplished by using siGENOME SMARTpool siRNAs obtained from Thermo Scientific (Waltham, Massachusetts). One nonsense-siRNA and siRNAs against ATM and ATR were used in the concentration of 10 nM. Transfection was performed with the transfection reagent Lipofectamine RNAimax (Invitrogen, Karlsruhe, Germany) according to the manufactures protocol. Protein knockdown was verified by western blot analysis 48 h after transfection. Cells were treated with the indicated doses of TMZ 24 h after transfection.

**Stable transfection of the fibroblasts with human MGMT cDNA**

Exogenous MGMT expression was accomplished by co-transfection of the wt and ATM mutated fibroblasts with 0.9 µg of the expression vector pSV2 containing the human MGMT cDNA, which has been described previously (13), and 0.1 µg of the pSV2neo plasmid for selection. For transfection the Effectene transfection kit (QIAGEN, Hilden, Germany) was used. After transfection cells were selected with 0.5 mg/ml G418 (Sigma-Aldrich,
Taufkirchen, Germany). Appearing clones were then picked and checked by western blots for MGMT expression. Transfected cells were maintained in medium containing 0.5 mg/ml G418. G418 was omitted from the medium during experiments.

**MTT assay**

The MTT-assay was done using the Thiazolyl Blue Tetrazolium Bromide obtained from Sigma-Aldrich (Taufkirchen, Germany). Briefly, cells growing in suspension were harvested and diluted in medium containing 1.67 mg/ml MTT solution. After completion of the MTT reaction, medium was removed and cells were incubated for 15 minutes in 0.04 M HCl/isopropanol. Absorbance was measured using a photometer (Tecan, Crailsheim, Germany) with a wavelength of 570 nm and a reference wavelength of 650 nm.

**Annexin V/PI double-staining**

For Annexin V/PI double-staining, cells were treated as described in the previous section, further incubated, harvested and re-suspended in 50 µl Annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, 0.1 % BSA, pH 7.4). 2.5 µl Annexin V-FITC reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was added and cells were incubated on ice for 15 minutes. Afterwards, DNA in the cells were stained with 1 µg/ml propidium iodide (PI) and analyzed with the FACS Calibur or FACS Canto II (BD Biosciences, Heidelberg, Germany) using the CellQuest Pro or the FACS Diva (BD Biosciences, Heidelberg, Germany) and WinMDI software (J. Trotter).

**BER assay**
The BER assay was performed as described previously (31). Synthetic oligonucleotides purchased from Eurofins (Ebersberg, Germany) and $^{32}$P-labeled ATP obtained from Perkin Elmer (Rodgau, Germany) were used.

**Alkaline comet assay**

At indicated time points after drug addition cells were harvested and imbedded in low melting point agarose on glass slides. Cells were lysed for 60 minutes with cold alkaline lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Sodium-Laurylsarcosinate, 1 % Triton X100, 10 % DMSO, pH 10). Before electrophoresis for 15 minutes at 25 V and 300 mA, cells were incubated in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13) for 20 minutes. Subsequently, the slides were neutralized (0.4 M Tris, pH 7.5) and dehydrated with 100 % ethanol. Dried slides were stained with 50 μg/ml PI and analyzed using the microscope (Nikon Microphot-FXA, Düsseldorf, Germany). Comet sizes were scored using the Komet v4.0 software (Kinetic Imaging, Liverpool, UK).

**Immune fluorescence**

At the indicated time points following drug addition, cells grown on glass cover slips were fixed with either 4 % formaldehyde or with 70 % Methanol/30 % Aceton and blocked with goat serum. Then cells were incubated overnight with anti-γH2AX primary antibody (1:1000, Millipore, Billerica, Massachusetts), washed with phosphate buffered saline and incubated with anti-mouse secondary antibody conjugated to Cy3 (1:600, Jackson Immuno Research, Suffolk, UK). Cells were stained with 1 µM To-Pro-3 (Invitrogen, Karlsruhe, Germany) and mounted on glass slides in Vectashield mounting medium (Vector Laboratories, Burlingame, California) and sealed using nail varnish. Microscopy was performed by laser scanning microscope (Carl Zeiss, Jena, Germany) with the ZEN 2008 software (Carl Zeiss, Jena, Germany).
Preparation of cell extracts

Whole cell protein extracts for western blots were prepared by two different protocols as described (32, 33). Protein concentration was determined with the Bradford assay (34).

Western Blotting

Western blot analysis was performed as published (35). The following primary antibodies were used: Anti-ATM (Cell Signaling Technology, Danvers, Massachusetts), Anti-ATR (Cell Signaling Technology, Danvers, Massachusetts), anti-cleaved caspase-7 (Cell Signaling Technology, Danvers, Massachusetts), anti-PARP-1 (BD Transduction Laboratories, Franklin Lakes, New Jersey), anti-MGMT (raised in the laboratory of B.K.), anti-pChk1 (Ser345) (Cell Signaling Technology, Danvers, Massachusetts), anti-pChk2 (Thr68) (Cell Signaling Technology, Danvers, Massachusetts), anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, California) and anti-Talin1 (Cell Signaling Technology, Danvers, Massachusetts). Horseradish-peroxidase-coupled and fluorophor-coupled secondary antibodies (Amersham Biosciences, Uppsala, Sweden & LI-COR Biosciences, Bad Homburg, Germany) were used for detecting the primary antibodies. Proteins were visualized using a chemiluminescence detection reagent (GE Healthcare, Munich, Germany) or the Odyssey system (LI-COR Biosciences, Bad Homburg, Germany).

Statistics

For statistical analysis the unpaired t-test was performed using the Prism v3 software (Graph Pad Software, San Diego, California). Values presented in graphs are the means of at least three independent experiments.
Results

ATM and ATR protect from cell death induced by methylating agents

In order to determine whether ATM and/or ATR plays a role in the protection of cells against methylating agent-induced cell death, cell pairs wild-type (wt) and mutated in ATM or ATR were compared in their response to methylating agents. Comparing the ATM and ATR protein levels in the cell lines (GM637 wt and ATs4 ATM mt, and AG09387 wt and DK0064 ATR mt respectively) it is obvious that the mutant cell lines express lower levels of the respective proteins than the wt cells (Fig. 1A). For all subsequent experiments, unless stated otherwise, cells were pre-treated with the MGMT inhibitor O6-BG to exclude any variability caused by differences in MGMT expression between the lines. The ATM (Fig. 1B) and the ATR defective cell line (Fig. 1C) were significantly more sensitive to TMZ than the corresponding wt. To substantiate these cytotoxicity data, the cell pairs were subjected to the AnnexinV/PI assay that determines apoptosis and necrosis after a genotoxic insult. Again, ATM (Fig. 1D) and ATR (Fig. 1E) defective cells were significantly more sensitive to TMZ than the corresponding wt. Data for the combined values are presented, the sum of apoptosis and necrosis, for the actual levels see supplemental Table 2. We checked another ATM mutated cell line (GM05849 ATM mt) as to its TMZ sensitivity, which again proved to be more sensitive than the wild-type (Fig. 1D). For further verification, the activation of caspase-7 (cleaved form) and PARP-1 cleavage was probed in the wt and ATM mutated fibroblasts; this time the cell pair was treated with the model methylating agent MNNG (Fig. 1F). The ATM defective cells show clearly more caspase-7 activation along with PARP-1 cleavage compared to the wt cells. Collectively, these data show that both ATM and ATR protect cells against cell death triggered by methylating agents.

ATR, and not ATM, plays the dominant role in protecting malignant glioma and melanoma cells against TMZ
As TMZ is used as first-line in the therapy of malignant glioma and metastasized melanoma, would the loss of ATM and/or ATR be of benefit in sensitizing these highly refractory cancers? To this end, ATM and ATR were transiently knocked-down in glioma (LN229) and melanoma (D03) cells (Fig. 2A). Knockdown decreased the ATM and ATR protein level in both cell lines by more than 80% (knockdown in glioma: 13 % for ATM, 19.6 % for ATR; knockdown in melanoma: 13.1 % for ATM, 16 % for ATR). This had a profound sensitization effect on the glioma line treated with TMZ (Fig. 2B). Both ATM and ATR knockdown sensitized the glioma line to TMZ, with the greatest benefit observed for the ATR knockdown. The results obtained for the ATM knockdown in the melanoma line were less impressive while ATR knockdown, similar to the glioma line, led to the most significant sensitization (Fig. 2C). No benefit was observed when both ATM and ATR were simultaneously knocked-down in the glioblastoma and melanoma lines (Fig. 2B and 2C); the sensitization was similar to ATR knockdown alone. Data for the combined values are presented, the sum of apoptosis and necrosis, for the actual levels see supplemental Table 3. From these results we conclude that down-regulation of ATR, and to a lesser extent ATM, is an effective strategy in sensitizing glioma and melanoma cells to TMZ.

O\(^6\)-methylguanine triggers the activation of ATM and ATR

In all previous experiments MGMT was inactivated in order to be able to determine the full extent of the contribution that O\(^6\)-MeG has on cell death induced by methylating agents. However, along with O\(^6\)-MeG methylating agents such as TMZ also induce N-methylpurines, and both of these types of lesions can exert cytotoxicity. Therefore, in order to determine whether ATM and ATR protects against cell death triggered by O\(^6\)-MeG or the N-methylpurines, cell lines that do not express adequate MGMT levels were stably transfected with a plasmid containing the cDNA of MGMT. Transfection gave rise to an increased MGMT protein level in the fibroblast lines, GM637 wt and ATs4 ATM mt and in the glioblastoma line LN229 (Fig. 3A). For the lymphoblastoid ATR cell pair, transfection with MGMT was
unnecessary as the lines AG09387 wt and DK0064 ATR mt contained similarly high MGMT protein levels. MGMT assays were performed to verify the activity of MGMT in the lymphoblastoid ATR cell pair and the increase in MGMT activity in the MGMT transfected cells (data not shown).

Having established an experimental system where cells are protected against cytotoxicity caused by O6-MeG, we were able to determine whether ATM and ATR protects against O6-MeG or the N-methylpurines. In the MGMT expressing ATM mt (Fig. 3B) and the ATR mt (Fig. 3C) lines methylating agents were unable to exert any significant cytotoxicity, while inactivation of MGMT with O6-BG restored their extreme sensitivity towards methylating agents. This experimental approach was extended to the glioma cell system. Similar to what was observed in the ATM and ATR mutants, over-expression of MGMT completely abolished TMZ-induced cytotoxicity, even when ATM and ATR was down-regulated by knockdown (Fig. 3D). Again, when these MGMT expressing cells were co-treated with O6-BG, cytotoxicity increased up to the level observed in the non-transfected cells (Fig. 3E). In summary, these data show that ATM and ATR protect against the specific killing lesion O6-MeG.

**ATM deficient fibroblasts show effective BER**

Because N-methylpurines are repaired by BER and ATM was reported to have an impact on this repair pathway, we investigated BER in ATM mutated cells using a functional *in vitro* assay. In this assay a 32P-labeled DNA oligonucleotide (39mer) containing a single apurinic site is cleaved (19mer) and after its repair re-ligated in the presence of whole cell protein extract of untreated cells. Both steps can be visualized by gel electrophoresis and autoradiography of the gel. In Fig. 4A a representative gel is shown, demonstrating cleavage and restoration of the DNA fragment with time after addition of the protein extracts of wt and A-T fibroblasts. Quantification of the restored DNA band revealed that A-T cells repaired the apurinic site slightly faster than wt cells (Fig. 4B). In line with the *in vitro* assay, the alkaline comet assay, which detects SSBs as BER intermediates, showed very effective BER in both
A-T and wt fibroblasts following MNNG treatment (Fig. 4C). With increasing time, decrease of tail moments reflecting ligation of SSBs occurred in both cell lines with the same kinetics. In conclusion, the induced N-methylpurines are repaired very efficiently by BER in wt and A-T fibroblasts. These data support the conclusion that non-repaired O6-MeG is the critical toxic lesion induced by O6-methylating agents such as TMZ.

**ATR, but not ATM, is required for H2AX, CHK1 and CHK2 phosphorylation following TMZ**

Once activated, ATM and ATR kinases phosphorylate critical downstream targets. The next step was therefore to determine which kinase plays the dominant role in signaling from O6-MeG adducts. To this end the number of γH2AX foci, the phosphorylated form of H2AX, was determined in the glioma cell line following ATM or ATR knockdown and TMZ treatment using immunofluorescence (Fig. 4D for representative images). Knockdown of ATM and ATR had opposing effects on γH2AX foci formation (Fig. 4E). Knockdown of ATM caused an increase in foci while knockdown of ATR caused a decrease. These data show that O6-MeG dependent H2AX phosphorylation is mediated by ATR and that ATM knockdown has an effect on the number of γH2AX foci that form, or the time it takes for the DSB to be repaired.

To further substantiate the role of ATR in the signaling from methylating agent induced DNA damage the activation of key ATM and ATR substrates, namely CHK2 and CHK1, were probed for. The immunoblots show a clear phosphorylation of CHK1 (Fig. 5A) and CHK2 (Fig. 5B) in the glioblastoma cells treated with TMZ. This activation was still present following ATM knockdown, showing that ATM is not required for the activation of CHK1 and CHK2 following TMZ treatment. Knockdown of ATR, however, prevented the phosphorylation of CHK1 and CHK2, demonstrating the requirement of ATR in the phosphorylation of these targets. In order to demonstrate that ATR is required for the downstream signaling from O6-MeG adducts induced by TMZ, these experiments were repeated with glioblastoma cells that over-express MGMT. Under these conditions no
activation of CHK1 or CHK2 could be shown. These data support the model where signaling from O6-MeG processed secondary DNA lesions (19) are caused by ATR and not ATM.

**Inhibition of CHK enhances temozolomide toxicity**

CHK1 and CHK2 trigger downstream cell death pathways (36). Therefore, we wished to determine whether inhibition of CHK1 and/or CHK2 impacts the sensitivity of LN229 and D03 cells to TMZ. As shown in Fig. 6A, the inhibitor of CHK1 (UCN-01) and the inhibitor of CHK2 significantly enhanced TMZ-induced cell death in the glioblastoma line. The sensitization effect was greater when CHK1 was inhibited. MGMT expression completely abrogated TMZ-induced cell death in glioblastoma cells both in the absence and presence of the CHK1 and the CHK2 inhibitor (Fig. 6B). In D03 cells the CHK1 inhibitor also led to increased cell death following treatment with TMZ. The CHK2 inhibitor, however, did not significantly change the TMZ-induced toxicity (Fig. 6C). The data indicates that both CHK1 and CHK2 can be involved in TMZ-induced cell death, with CHK1 being the more important kinase, and that O6-MeG is the critical signaling-triggering lesion.

**Discussion**

The DNA-methylating agent TMZ is used in the chemotherapy of malignant glioma and metastasized melanoma (37, 38). It is well established that its anticancer activity rests on the formation of the minor DNA adduct O6-MeG, which is converted by DNA replication and mismatch repair into DSBs (39) that trigger apoptosis, necrosis and autophagy (16, 40). TMZ has been shown to induce the DNA damage response (DDR), which rests on the activation of the PI3 kinases ATM, ATR (18) and presumably also DNA-PKcs (41), and phosphorylation of downstream targets such as histone 2AX, CHK1 and CHK2 (16, 18). This finally leads to activation of the apoptotic pathway in malignant glioma (41) and melanoma cells (33, 42).
Although it is likely that all of these responses are triggered by O⁶-MeG, a systematic study is lacking as to the importance of ATM and ATR in the O⁶-MeG triggered TMZ response.

Here, we addressed the question of the contribution of ATM and ATR in the killing response of glioblastoma and malignant melanoma cells to TMZ, using different cell models. First, we show that cells obtained from A-T and Seckel syndrome patients are hypersensitive to TMZ, responding with a prompt cell death induction. The ATM data confirm a previous study with mouse ATM knockout cells that showed that ATM knockout cells are more sensitive than the corresponding wild-type to the O⁶-methylating agent MNNG (22). In ATR mutated cells the O⁶-MeG response has not been studied to date.

Having shown that both ATM and ATR determine the resistance of cells to TMZ, we extended the study to glioblastoma and malignant melanoma cells, using the well-characterized lines LN229 (29) and D03 (28), respectively. This was accomplished by down-regulation of ATM or ATR in these cells and determining the impact on their sensitivity to TMZ. Knockdown of either ATM or ATR greatly enhanced the sensitivity of LN229 and D03 cells to TMZ, with a significantly stronger sensitizing effect if ATR was down-regulated. Simultaneous down-regulation of ATM and ATR had no additional effects on the killing response. The data indicate that ATM and, even more, ATR, are targets for improving the anticancer effect of TMZ. We should stress that the TMZ doses applied in the experiments are in the therapeutic range, i.e. up to 100 µM, which corresponds to the drug serum level during therapy (43).

ATM and ATR activate CHK2 and CHK1, respectively, which are involved in triggering cell death pathways (44). This study revealed that TMZ treatment results in the activation of both CHK2 and CHK1. Since MGMT expression nearly completely abrogated CHK2 and CHK1 activation we conclude that the specific DNA damage O⁶-MeG is responsible for triggering this response. Therefore we wondered whether inhibition of either CHK2 or CHK1 is effective in enhancing the killing effect of TMZ. Pharmacological inhibition of CHK1 and to some extent also CHK2 enhanced the cell killing response to TMZ. This enhancement was
completely abolished if MGMT was expressed in the cells, which substantiates the model that O\textsuperscript{6}-MeG DNA adducts are the primary lesions triggering the ATM-ATR-CHK1-CHK2 pathways.

According to the “classical” model, ATM is triggered by free DSBs while ATR becomes activated by blocked replication forks resulting from adducts induced by UV light and chemical DNA damaging agents (45, 46). We, therefore, supposed that ATM does not play a role in the TMZ-induced DDR as the drug does not directly induce free DSBs. However, O\textsuperscript{6}-MeG/thymine mispairs processed by MMR cause secondary lesions that severely block replication in the 2\textsuperscript{nd} cell cycle (19), which in turn lead to the formation of DSBs (19). This scenario supports the model that blocked replication forks activates ATR, and that ATM is activated as a secondary consequence of replication inhibition giving rise to DSB formation at blocked replication forks. We should note that ATR and ATM do not exclusively activate CHK1 and CHK2, respectively, but that there is crosstalk between both pathways (9-11). Therefore, both CHK1 and CHK2 activation in glioma and melanoma cells following TMZ treatment is likely accompanied by ATR, which was shown here to be the case.

Interestingly, we observed that knockdown of ATM enhanced the amount of TMZ-induced γH2AX foci whereas knockdown of ATR reduced the γH2AX foci level. We explain this finding on the basis of the supposition that ATR is the primary kinase activated by O\textsuperscript{6}-MeG induced secondary lesions that trigger the phosphorylation of H2AX. Therefore, lack of ATR abolishes H2AX phosphorylation. In contrast, ATM might be acting as a repair stimulator of DSBs resulting from O\textsuperscript{6}-MeG/T mispairs, without being required for the initial recognition of stalled replication forks, which arise after initiation of O\textsuperscript{6}-MeG-induced futile MMR cycles.

If ATR and ATM have an impact on the sensitivity of glioblastoma and malignant melanoma cells to O\textsuperscript{6}-methylating drugs (TMZ, and also dacabazine and procarbazine that need metabolic activation by liver cytochrome P450), it is pertinent to conclude that the
expression level of these kinases as well as their mutation status is important for the therapeutic response. Because MGMT abolishes the increased sensitivity given by the impaired function of ATM or ATR, the therapeutic benefit is limited to patients with tumors that lack MGMT expression. In malignant glioma specimens a reduced level of expression (47), variable expression (48) and high expression (49) of ATM has been reported. In metastasized melanoma, high ATM expression compared to normal cells was reported (50). With respect to ATR, down-regulation of expression was found in malignant gliomas compared to normal brain tissue (51). Regarding the expression of checkpoint kinases, glioblastoma (52) but not sporadic glioma (53) were shown to carry mutations in CHK2. It was also shown that CHK1 and CHK2 expression was down regulated by promoter hypermethylation in glioma (51). Mutations in both proteins were also found in metastasized melanoma cells (54).

ATM expression was related to the therapeutic outcome in glioblastoma patients following temozolomide treatment in combination with ionizing radiation (55). CHK2 gene polymorphism also correlated with prognosis of glioblastoma patients receiving radiochemotherapy (56). Most studies pertained to the response of glioblastoma cells to radiation therapy. Thus, loss of ATM and CHK2 were reported to provoke radioresistance in a mouse glioma model (57) whereas pharmacological inhibition of CHK1 and CHK2 reverted radioresistance in glioma cells in vitro (58). Since the radiation response of cells, including the activation of death pathways, is clearly different from chemical agents, translation of this data to TMZ-triggered responses is not possible. Previous studies revealed that CHK1 inhibition increases TMZ cytotoxicity (59), which is in line with our data.

This is the first study in which the role of ATR and ATM, CHK1 and CHK2 triggered by a specific DNA damage, O6-MeG, in the killing response of malignant glioma and melanoma cells to TMZ was investigated comparatively. We identified both pathways to be involved, with a dominance of the ATR pathway. Therefore, inhibition strategies of this pathway (59, 60) together with inactivation of MGMT (61) and homologous recombination mediated by
Rad51 and BRCA2 (30) might be reasonable strategies for improving the effectiveness of treatment of malignant glioma and melanoma cells with temozolomide and other O\textsuperscript{6}-methylating treatment modalities.

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References


Figure legends

Figure 1: Induced cellular toxicity following exposure to the genotoxic agent TMZ and MNNG in wt, ATM and ATR mutated cells. A, ATM and ATR expression determined by western blot analysis of whole cell protein extracts of fibroblasts and lymphoblastoid cells. B, and C, cell viability analyzed with the MTT assay 72 h after exposure to TMZ with indicated concentrations in fibroblasts (B) and lymphoblastoid cells (C). D, and E, induced overall cell death determined with flow cytometric analysis of Annexin V/PI double-stained cells 72 h after TMZ-treatment with indicated concentrations in human fibroblasts (D) and lymphoblastoid cells (E). The overall cell death was calculated by combining the apoptotic and necrotic fraction. F, cleavage of Caspase-7 and PARP-1 after treatment with 10 µM MNNG investigated by western blot analysis of whole cell protein extracts of fibroblasts. To inactivate MGMT cells were co-treated with 10 µM O6-BG in all experiments.

Figure 2: Influence of ATM and ATR protein knockdown on induced cellular toxicity following exposure to the genotoxic agent TMZ in glioblastoma and melanoma cells. A, residual ATM- and ATR-expression determined by western blot analysis of whole cell protein extracts of glioblastoma and melanoma cells 48 h after transfection with 10 nM control siRNA and siRNAs against ATM or ATR. B, and C, induced overall cell death investigated with flow cytometric analysis of Annexin V/PI double-stained cells 144 h after 100 µM TMZ treatment in glioblastoma (B) and melanoma cells (C). Cells were transfected transiently with 10 nM siRNAs. TMZ treatment was performed 24 h after protein knockdown. MGMT was inactivated by co-treatment of the cells with 10 µM O6-BG. To keep the overall siRNA concentration constant cells transfected with 20 nM control siRNA were used to compare with the cells which received the ATM/ATR double knockdown.
Figure 3: Influence of MGMT on MNNG- and TMZ-induced toxicity. A, expression of MGMT determined by western blot analysis of whole cell protein extracts of fibroblasts and glioblastoma cells, which were manipulated or not manipulated by stable transfection with human cDNA for MGMT, and lymphoblastoid cells. B and C, protective role of MGMT against MNNG-induced toxicity analyzed with Annexin V/PI double-stained cells 72 h after 10 µM MNNG treatment in fibroblasts (B) following transfection with MGMT cDNA and lymphoblastoid cells (C). Cells were either co-treated or not co-treated with 10 µM O6-BG. D and E, protective role of MGMT against TMZ-induced toxicity analyzed with Annexin V/PI double-stained cells 144 h after 100 µM TMZ treatment. Glioblastoma cells were transfected stably with cDNA for MGMT and transiently with 10 nM control siRNA or siRNA against ATM and ATR. To keep the total amount of siRNA constant, control cells which are compared to cells received the ATM/ATR double knockdown are treated with 20 nM control siRNA. TMZ was applied 24 h after protein knockdown either without (D) or with O6-BG co-treatment (E).

Figure 4: BER capacity of wt and ATM mutated fibroblasts and γH2AX foci formation in glioblastoma cells. A, representative gel of the in-vitro BER assay. The cleavage and reconstitution of a synthetic DNA-oligonucleotide containing a single apurinic site in the presence of whole cell protein extracts is shown as a function of time. B, quantification of the relative BER capacity by comparing the intensity of the restored DNA-oligonucleotide with the control at indicated time points. C, detection of DNA SSBs by the alkaline comet assay following a one-hour pulse of 15 µM MNNG. D, images of representative glioblastoma cell nuclei, which were investigated by immunofluorescence 48 h following 1 µM TMZ treatment. 24 h before drug treatment cells were transfected transiently with siRNAs (control, ATM or ATR) with a final concentration of 10 nM. To deplete MGMT, cells were co-treated with 10 µM O6-BG. To-Pro-3 stained cell nuclei are shown in blue, γH2AX foci in red. E, Quantification of γH2AX foci shown in C.
Figure 5: Phosphorylation of ATM and ATR downstream targets CHK1 and CHK2. A, and B, phosphorylation of CHK1 (A) and CHK2 (B) was analyzed by western blot analysis 48 h and 96 h after treatment with 100 µM TMZ in glioblastoma cells deficient or proficient for MGMT expression. The analyses were performed after transient ATM or ATR knockdown by transfection with 10 nM siRNAs. For comparison glioblastoma cells were transfected with 10 nM control siRNA. TMZ treatment was performed 24 h after siRNA transfection. ERK2 serves as a loading control. To inhibit any non-detectable MGMT molecules MGMT deficient cells were co-treated with 10 µM O6-BG. MGMT proficient cells were not exposed to O6-BG.

Figure 6: Influence of CHK1 and CHK2 inhibition on TMZ-induced cytotoxicity. A, cell death induced by TMZ in LN229 glioblastoma cells that do not express MGMT. B, cell death induced by TMZ in LN229 stably expressing MGMT. Data are expressed relative to the non-treated (without CHK inhibitor) control. For the MGMT stably expressing LN229 cells, the actual apoptosis values for control, CHK2-Inh. II and UCN-01 treated samples were 1.5, 0.9 and 2.2% respectively. C, TMZ-induced death of D03 melanoma cells. Cell death was investigated by flow cytometric analysis of Annexin V/PI double-stained cells 144 h following exposure to 100 µM TMZ. CHK1 was inhibited by UCN-01, CHK2 by CHK2-inhibitor II. Treatment with the inhibitors was done 24 h after TMZ treatment (150 nM for UCN-01 and 10 µM for CHK2-inhibitor II). LN229 and D03 cells, but not the MGMT transfected lines, were co-treated with 10 µM O6-BG.
Fig. 2

(A) Western blot analysis showing the relative expression of ATM, ATR, and Talin proteins in LN229 and D03 cells treated with siCON or siATM. The relative expression levels are indicated as percentages: ATM (LN229: 100 vs. 13; D03: 100 vs. 13.1), ATR (LN229: 100 vs. 19.6; D03: 100 vs. 16), and Talin (LN229: 100 vs. 100; D03: 100 vs. 100).

(B) Bar graph showing the induced cell death (%) in LN229 cells treated with various siRNAs and 100 μM TMZ. The treatments include non-transfected, control siRNA, ATM siRNA, ATR siRNA, and ATM/ATR siRNA groups. The data are presented as mean ± SD, with statistical significance indicated by *** (p < 0.001), ** (p < 0.01), * (p < 0.05), and ns (not significant).

(C) Bar graph showing the induced cell death (%) in D03 cells treated with various siRNAs and 100 μM TMZ. The treatments include non-transfected, control siRNA, ATM siRNA, ATR siRNA, and ATM/ATR siRNA groups. The data are presented as mean ± SD, with statistical significance indicated by *** (p < 0.001), ** (p < 0.01), * (p < 0.05), and ns (not significant).
Fig. 3

A

B

C

D

E

MGMT

ERK2

β-actin

10 μM MNNG

10 μM MNNG + O6-BG

Induced cell death (%)

GM337 wt

ATs4 ATM mt

Induced cell death (%)

AG09387 wt

DK0064 ATR mt

Induced cell death (%)

Non-transfected

Control siRNA

ATM siRNA

ATR siRNA

ATM/ATR siRNA

100 μM TMZ

100 μM TMZ + O6-BG

LN229-MGMT-12

Induced cell death (%)

Non-transfected

Control siRNA

ATM siRNA

ATR siRNA

ATM/ATR siRNA

**

* ns

***

Significant differences indicated by asterisks.
Fig. 4
Fig. 6
Molecular Cancer Therapeutics

Contribution of ATM and ATR to the resistance of glioblastoma and malignant melanoma cells to the methylating anticancer drug temozolomide


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