Molecular dosimetry of temozolomide: Quantification of critical lesions, correlation to cell death responses and threshold doses

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Running title: Molecular dosimetry of temozolomide
Abstract

Temozolomide is a DNA-methylating agent used in cancer chemotherapy, notably for glioblastoma multiforme (GBM) where it is applied as a front-line drug. One of the DNA alkylation products is the minor lesion O6-methylguanine (O6MeG), which is responsible for nearly all genotoxic, cytotoxic and cytostatic effects of TMZ in the low-dose range relevant for cancer therapy. Here, we addressed the question of how many O6MeG adducts are required to elicit cytotoxic responses. Adduct quantification revealed that O6MeG increases linearly with dose. The same was observed for DNA double-strand breaks (DSBs) and p53ser15. Regarding apoptosis, hockeystick modeling indicated a possible threshold for A172 cells at 2.5 µM TMZ, while for LN229 cells no threshold was detected. Cellular senescence, which is the main cellular response, also increased linearly, without a threshold. Using a dose of 20 µM, which is achievable in a therapeutic setting, we determined that 14,000 adducts give rise to 32 DSBs (γH2AX foci) in A172 cells. This leads to 12% cell death and 35% of cells entering senescence. In LN229 cells, 20 µM TMZ induced 20,600 O6MeG adducts, 66 DSBs (γH2AX foci), 24% apoptosis and 52% senescence. The linear dose-response and the genotoxic and cytotoxic effects observed at therapeutically relevant dose levels makes it very likely that the TMZ target concentration triggers a significant cytotoxic and cytostatic effect in vivo. Despite a linear increase in the O6MeG adduct level, DSBs and p53 activation, the low curative effect of TMZ results presumably from the low rate of apoptosis compared to senescence.

Introduction

Temozolomide (TMZ) is a DNA-methylating agent frequently used in cancer therapy [1]. It is applied in first-line therapy for high-grade gliomas, including astrocytoma (WHO grade 3) and glioblastoma multiforme (glioma WHO grade 4) [2] and in some other cancers [3]. TMZ is a triazene derivative that does not need metabolic activation. It decomposes spontaneously yielding 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) and, in a second step, 5-aminoimidazole-4-carboxamide (AIC) and monomethyl hydrazine, which finally methylates DNA at various sites. As revealed by studies with DNA repair mutants and isogenic cell lines [4, 5], the main genotoxic and cytotoxic target of TMZ is the nuclear DNA. Similar to other \( S_N1 \) alkylating agents, at least 12 nucleophilic sites are subject of methylation [6]. The major methylation products are \( N \)-methylpurines such as \( N7 \)-methylguanine, \( N3 \)-methylguanine and \( N3 \)-methyladenine (comprising about 80% of total alkylation), while \( O \)-methylpurines are less frequent. Thus, \( O^6 \)-methylguanine (\( O^6 \)MeG) accounts for only 7% of the total DNA methylation [6].
Although produced in minor amounts, O\textsuperscript{6}MeG is the main mutagenic, carcinogenic, genotoxic and cytotoxic lesion (reviewed in [7]). It is also responsible for autophagy and cellular senescence, which are endpoints induced by TMZ concomitant to apoptosis [8].

O\textsuperscript{6}MeG is repaired by the suicide enzyme O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) in a damage reversal reaction [9]. If cells are repair competent, O\textsuperscript{6}MeG is quickly removed from DNA while in repair-incompetent cells, O\textsuperscript{6}MeG persists. Since O\textsuperscript{6}MeG is not eliminated by spontaneous hydrolysis, it can be transmitted even to the next cell generation, exerting long-term effects [10]. The MGMT gene is a subject of intensive genetic and epigenetic regulation and therefore cells and tissues vary considerably in their repair capacity [11-13]. High expression levels were observed in liver and the lowest in brain [13]. Brain tissue of rats is completely devoid of MGMT, leading to persistence of O\textsuperscript{6}MeG, which is the reason for neurotropic carcinogenesis following exposure with S\textsubscript{n}1 methylating agents such as N-nitroso-N-methyl-urea [14]. The human brain expresses MGMT, although at low levels compared to other organs [15, 16]. About 40% of human brain cancer (glioma grade 3 and 4) show MGMT promoter hypermethylation, which is related to MGMT downregulation, and 17% of glioblastoma multiforme (GBM) are completely devoid of MGMT repair activity as determined in an enzymatic assay [17]. Thus, promoter-methylated GBM are responsive to TMZ therapy [18-20].

In MGMT repair-competent cells, higher doses of TMZ are required to be cytotoxic, which results from non-repaired N-alkylation lesions that interfere with the replication machinery [21]. These lesions are repaired by base excision repair (BER) and ALKB homologous proteins (ALKBH) [7] and, therefore, they do not contribute to cytotoxicity if O\textsuperscript{6}MeG is left in the DNA. However, if at high TMZ dose levels the N-alkylation repair system is saturated, these lesions contribute to cell death as well. The doses of TMZ in a therapeutic setting are very likely too low to achieve cell death resulting from N-alkylation products. Therefore, with a serum concentration of ~50 µM TMZ, the O\textsuperscript{6}MeG adducts are the key players inducing tumor cell death.

The mechanisms of O\textsuperscript{6}MeG triggered cell death responses have been intensively investigated [22]. In brief, a widely accepted model claims that O\textsuperscript{6}MeG is a mutagenic mispairing lesion resulting in mismatches with thymine that are subject to mismatch repair (MMR). Reinsertion of thymine during MMR causes a futile MMR cycle, resulting in gaps in the DNA that finally give rise to DNA replication blockage and the formation of replication-mediated DNA double-strand breaks (DSBs). This occurs in the post-treatment cell cycle, which is compatible with the time-course of apoptosis and DSB formation [23]. Results obtained with synchronized cells confirmed this model [10]. It is further known that DSBs induced by the processing of O\textsuperscript{6}MeG/thymine trigger complex DNA damage response pathways, which are activated primarily by ATR, and secondary by ATM, and downstream CHK1 and CHK2, respectively [24] as well as
activation of the HIPK2-p53 axis [25]. In this scenario, the following factors determine drug resistance: MGMT, MMR, the proliferation level, DNA damage response (DDR) activation and DSB repair by homologous recombination. O\textsuperscript{6}MeG is not only an apoptosis-inducing damage, it also triggers with high efficiency cellular senescence in glioblastoma cells [8]. The pathways involved were previously described [8, 26, 27].

Patients suffering from GBM, which make up ~70% of high-grade malignant gliomas, have a dismal prognosis [2]. Standard therapy includes resection of the tumor followed by radiotherapy and concomitant chemotherapy with TMZ [28]. TMZ is also prescribed in the recurrent situation and as maintenance therapy, and is well tolerated even if treatment occurs daily over long periods of time [29]. In view of the bad prognosis and weak therapeutic efficiency of TMZ, better knowledge of the action of this anticancer drug is highly desirable. Given the known mechanism of action of TMZ, some questions arise which are a matter of debate [27, 30]. Firstly, do GBM cells die (by apoptosis or other forms of cell death) following TMZ treatment or does the drug exert only a cytostatic effect? Secondly, are the doses used for patient's treatment high enough to kill cancer cells without having toxic side effects on non-cancer cells? This question relates to the paradigm that low doses of a genotoxic anticancer drug are ineffective and that presumably thresholds exist below which cytotoxicity does not occur. And thirdly, how many O\textsuperscript{6}MeG adducts are required for eliciting a specific effect such as apoptosis, necrosis and senescence and can this level be achieved in vivo?

To address these fundamental questions in the molecular dosimetry of TMZ, we measured the amount of O\textsuperscript{6}MeG induced by TMZ in established GBM cell lines and related this to levels of DSBs, apoptosis, necrosis, overall cell death and cellular senescence. The data revealed that the amount of O\textsuperscript{6}MeG following TMZ exposure, similar to DSBs, increased linearly with dose. Likewise, cell death and senescence tracked with the formation of these damage products. Finally, we found that even at low dose levels genotoxic, cytotoxic and cytostatic effects were induced by TMZ. The amount of lesions inducing a particular effect was determined.

**Material and methods**

**Cell lines and culture conditions**

The human glioblastoma cell lines LN229 and A172 were purchased from American Type Culture Collection (ATCC), expanded, stored frozen in batches and kept in culture only for limited time periods (maximally 2 month). LN229-MGMT (clone 12) was generated by stable transfec-
tion of LN229 with a human MGMT cDNA expression vector as described previously [8, 31]. Cells were cultured in DMEM with Glutamax (Gibco, Life Technologies Corporation, Paisley, UK) and 10% fetal calf serum at 37°C in a humidified 5% CO₂ atmosphere and routinely checked for mycoplasma contamination using a kit (Venhör GeM Classic) from Minerva Biolabs (Berlin, Germany). Cells were seeded 48 h before treatment, when they reached exponential growth. For short-term experiments (harvest 3 days after treatment), 2x10⁵ cells were seeded per 5-cm or 6-well dish (in 5 or 4 ml medium, respectively), for long-term experiments (apoptosis and senescence following TMZ) 1x10⁵ cells were seeded per dish. Under these conditions, cells were kept in exponential growth for the whole experimental period.

**Drugs and drug treatment**

TMZ obtained from Dr. Geoff Margison (University of Manchester, UK) was dissolved in DMSO (150 mM stock) and stored in batches at -80°C until use. Immediately before use, it was diluted 1:10 in sterile distilled water and added to the cell culture medium at the desired final concentration. For low final concentrations (≤10 µM) the solution was further diluted with distilled water to a 1 mM stock solution. Water-diluted stocks were used only once. The amount of DMSO in the medium did not exceed 0.05% and was without any cytotoxic effect (controls).

**Quantification of O⁶MeG adducts**

O⁶MeG adduct quantification was performed by measuring O⁶-methyl-deoxyguanosine (O⁶MedG), which was normalized to the deoxyguanosine (dG) content [32], according to a modified method as recently described in detail [33]. Briefly, exponentially growing cells were treated with TMZ and cells were harvested at the time points indicated by trypsinization. Cells were taken up in complete medium, washed with PBS before the cell pellet was stored frozen at -80 °C. DNA from cell pellets with a defined cell number was isolated using the QIAamp® DNA Mini Kit (QIAGEN®, Switzerland). Briefly, lysis buffer, proteinase K and RNase A were added and cell pellets were incubated for 20 min at 56 °C, 1400 rpm on a ThermoShaker. Afterwards, ethanol was added and DNA was purified and extracted on spin columns applying washing buffers and MilliQ for elution according to the manufacturer’s instruction. Internal labelled standards were added to the sample and DNA was enzymatically hydrolyzed, yielding free nucleosides. Following further sample clean-up by solid phase extraction, O⁶MedG was quantified by reversed-phase nano-liquid chromatography electrospray ionization high-resolution tandem mass spectrometry (nanoLC-ESI-HRMS²). The results were expressed as number of O⁶MeG per 10⁷ nucleotides (nt). The results were then converted to O⁶MeG per cell. The calculation rests on the measured DNA content of 5.11 pg DNA per cell in the exponentially growing A172 population, and 8.22 pg DNA per cell in the exponentially growing LN229 population. It further rests on the
relationship of 1 pg DNA corresponding to $10^9$ bp (2x$10^9$ nt), which gives rise to the following equation: $O^9MeG/10^7$nt x pg DNA/cell x 2 x $10^9$nt/pg.

**Quantification of apoptosis and necrosis**

The fraction of apoptotic and necrotic cells was determined using annexin V and propidium iodide (A/PI) staining of cells and measured by flow cytometry. For analysis, trypsinized cells including the supernatant were collected, transferred into 15 ml falcon tubes, washed with PBS, and stored on ice. For labeling, cells were incubated for 15 min at RT in 50 µl 1x annexin binding buffer containing 2.5 µl annexin V/FITC (MiltenyiBiotec GmbH, BergischGLadbach, Germany). For PI staining, 10 µl PI from a 1mg/ml stock solution (Sigma-Aldrich, Steinheim, Germany) and annexin binding buffer were added to each sample. Cells were incubated for additional 10 min on ice. Cells were kept in the dark until measurement. Data acquisition was performed using the FACS Canto II flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany) and the data was analyzed using the Flowing Software 2 program (PerttuTerho, Turku Center for Biotechnology, University of Turku, Finland). Apoptotic cells were defined as Annexin V+ / PI- cells, whereas necrotic cells were defined as Annexin V+ / PI+ cells. A representative plot showing the Annexin V+ / PI- and Annexin V- / PI+ fractions is shown in supplement (Fig. S1).

**Quantification of cellular senescence**

Induced senescence was determined by the amount of cellular SA-β-galactosidase. To inhibit endogenous β-galactosidase activity, cells were pre-incubated with 300 µM chloroquine for 30 min in the incubator after which C12:FDG was added to each sample at a final concentration of 33 µM. Following a 90 min incubation period, cells were washed once with cold PBS and collected by trypsinization. Cell pellets were washed again with cold PBS, resuspended in cold PBS and stored on ice. After the addition of chloroquine, cells were kept in the dark up to harvest. Data acquisition was performed using the FACS Canto II flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany) and the data was analyzed using the Flowing Software 2 program (PerttuTerho, Turku Center for Biotechnology, University of Turku, Finland). Untreated cells served as control. The cell population showing SA-β-GAL mediated staining higher than the control was defined as senescent.

**Quantification of γH2AX and 53BP1 foci**

The γH2AX and 53BP1 foci assays were conducted essentially as described [34]. Evaluation of γH2AX and 53BP1 stained nuclei of cells grown on coverslips occurred by LSM from a single focus plane. At least 100 cells were assessed per experiment and all experiments were performed three times independently. Antibodies used were γH2AX (1:500, rabbit, Cell Signaling
Technology, mAb #9718S) combined with Cy3 goat-anti-rabbit (1:500, Abcam, ab97075) and 53BP1 (1:500, mouse, Sigma Aldrich, MAB 3802) combined with Alexa Fluor 488 goat-anti-mouse (1:500, Thermo Fisher Scientific Invitrogen, A11017). The foci were counted with the software ImageJ (Wayne Rasband, NIH).

Statistics and mathematic assessments

Data are presented as mean of at least three independent experiments ±SEM and compared by unpaired t-test with Welch’s correction. The calculation of O\textsuperscript{6}MeG levels per cell was described above.

Results

Amount of O\textsuperscript{6}MeG induced by TMZ

In order to relate phenotypic effects of TMZ exposure with primary adduct formation, first we determined the amount of O\textsuperscript{6}MeG formed in glioblastoma cells following treatment with TMZ. The drug has a half-life of 1.9 h in serum [35] and, therefore, even chronic exposure can be considered as pulse-treatment. To establish the optimal time of treatment for determining the alkylation level, we exposed cells to TMZ for different durations. As shown in Fig. 1A, O\textsuperscript{6}MeG was induced in LN229 cells, which are MGMT repair incompetent, with a maximum level 3-4 h after addition of 50 µM TMZ to the medium and did not further increase due to the short half-life of the drug in the medium. In a separate series of experiments, we compared LN229 with LN229 stably transfected with human MGMT (LN229-MGMT) and observed that the amount of O\textsuperscript{6}MeG was significantly lower in MGMT-expressing cells even 3 h after the onset of treatment (Fig. 1B), which was expected as MGMT performs a fast repair reaction [36].

After having established the optimal treatment time for adduct quantification to be 3 h, we determined the dose-response relationship for the induction of O\textsuperscript{6}MeG. The amount of O\textsuperscript{6}MeG in the DNA of A172 cells increased as a linear function of TMZ dose (Fig. 1C). Similar data were obtained for LN229 (Fig. 1D). Thus, a dose of 50 µM TMZ induced 34 and 31 O\textsuperscript{6}MeG/10\textsuperscript{7} nucleotides in A172 and LN229 cells, respectively (for a direct comparison of A172 and LN229 see supplement Fig. S2). A172 and LN229 do not express MGMT due to MGMT promoter hypermethylation and are unable to repair O\textsuperscript{6}MeG [37]. Therefore, we used MGMT transfected cells and found that O\textsuperscript{6}MeG could not be detected if cells were treated with doses below 50 µM (Fig. 1D), indicating efficient and complete repair of the damage in this low dose range. At high dose levels, however, a small increase in O\textsuperscript{6}MeG was recorded (Fig. 1D), indicating increasing saturation of repair activity.

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**DNA double-strand breaks induced by TMZ**

A highly sensitive and generally accepted method of detecting DSBs is based on the phosphorylation of histone 2AX (H2AX) around the DNA break, which is catalyzed by damage-activated ATR and ATM proteins, resulting in immuno-detectable nuclear foci (γH2AX). At the same time, 53BP1, an accessory protein supporting the repair of DSBs, becomes phosphorylated and recruited to the DSB sites. Thus, γH2AX foci and colocalized 53BP1 foci are considered to be reliable markers of DSBs. Therefore, we quantified γH2AX and 53BP1 foci (for representative images see Fig. 2A) in cells exposed to TMZ. The dose-response curves shown in Fig. 2B-D demonstrate a linear increase of foci without a threshold. Thus, already a low dose of 5 µM TMZ gave rise to a significant increase in the DSBs, as measured 72 h after TMZ treatment in A172 cells.

**Induction of the DNA damage response: p53 and p21**

It is well established that DSBs trigger the DNA damage response (DDR), which targets downstream p53, phosphorylating p53 at serine 15. This phosphorylation leads to nuclear translocation, trimerization and activation of p53 as transcription factor. One of the p53 target genes is p21, which is a key player in cell cycle regulation. As shown in Fig. 3, p53ser15 and concomitantly p21 are upregulated dose-dependently until reaching a saturation level, which is achieved with a dose of about 10-15 µM TMZ (Fig. 3A for a representative western blot, Fig. 3B for total p53 and Fig. 3C and 3D for the dose-response curves of p53ser15 and p21).

Mathematical analysis of the linear dose range (0-15 µM) did not reveal a no-effect threshold dose (Fig. 3E and 3F). It should be noted that total p53 protein was not enhanced (Fig. 3B), which is due to the fact that p53 is mutated in A172 cells, leading to its stabilization without having impact on its trans-activating activity.

**Induction of apoptosis and necrosis by TMZ**

As previously shown, the induction of DSBs triggered by O^6^MeG precedes the activation of cell death pathways, which is a late response following DNA damage [23]. The time course of apoptosis, necrosis, total cell death and induced cellular senescence of A172 cells is shown in Fig. 4, demonstrating that cell death by apoptosis (Fig. 4A) and necrosis (Fig. 4B) starts at 96 h significantly above the control level and increases thereafter continuously. The onset of cellular senescence was observed earlier (Fig. 4D; significant increase 48 h after treatment p<0.05). Based on these data, we decided to measure for the influence of TMZ dose on these endpoints 120 h after addition of TMZ to the medium.

We measured the rate of apoptosis, necrosis and total cell death upon treatment with TMZ using a dose range of up to 50 µM. We used defined seeding conditions to ensure expo-
ential growth over the whole post-exposure period. As shown in the supplementary data (Fig. S3), apoptosis increased up to a level of max. 8% and then reached saturation at a dose of 20 µM. In contrast, necrosis increased over the whole dose range linearly up to a level of 7% at 50 µM. Since necrosis was a minor trait, the total cell death level reached saturation at 20 µM. Induced senescence increased up to 30-40% and reached a saturation level at 20 µM (Fig. S3).

Having established the linear dose range, we conducted further experiments using the dose range of 0-20 µM TMZ. As shown in Fig. 4 (panels E,F,G), apoptosis, necrosis and total cell death increased as a linear function of dose. An analysis of the data on the basis of the hockey stick model [38] revealed a possible threshold at 2.5 µM for the endpoint apoptosis, while for necrosis a significant threshold was not detected (see supplement, Fig. S4).

**Cellular senescence induced by TMZ**

Similar experiments were performed with the endpoint cellular senescence, which was quantified by C12FDG-flow cytometry. The level of senescence increased again as a linear function of dose. For senescence no threshold was observed (Fig. 4H), which was confirmed by hockey stick modeling of the data (supplement, Fig. S5).

**Correlation between O6MeG and DSBs, cell death and senescence**

Next, we wished to know how many O6MeG lesions per cell give rise to a DSB and cytotoxic effects. To this end, we recalculated the amount of O6MeG per unit DNA into O6MeG adducts per cell. The DNA content per cell was determined based on the amount of DNA extracted from a given number of cells in an exponentially growing population; 5.11 and 8.22 pg DNA per cell for A172 and LN229, respectively. The dependence of γH2AX, 53BP1 and colocalized foci with the level of induced O6MeG adducts per cell are shown in Fig. 5A, Fig. 5B and Fig. 5C, respectively, for a dose range up to 25 µM TMZ. Assuming a 1:1 relation between γH2AX foci and DSBs, we estimated for A172 cells that 490 O6MeG adducts per cell are required to induce one DSB (calculated from the slope of regression). Assuming that one γH2AX/53BP1 colocalized focus corresponds to one DSB, about 3,350 adducts are required for inducing one DSB.

The dose-response relationships for apoptosis, necrosis, total cell death and senescence with O6MeG adducts per cell are presented in Fig. 5 (panels D-G) for a dose range up to 20 µM. From this we calculated that an increase by 1% in the population of apoptotic cells requires 2,560 O6MeG adducts per cell, of necrosis 5,220 O6MeG and total cell death 1,580 O6MeG per cell. A 1% increase of cells undergoing senescence requires only 440 O6MeG adducts per cell. It is important to note that senescence is the major endpoint triggered by O6MeG lesions (7.5%
apoptosis and 3.5% necrosis compared to 35% senescence 120 h after TMZ treatment) and, therefore, a lower amount of $O^\delta$MeG adducts is required to trigger this response.

$O^\delta$MeG and responses triggered in LN229 cells

The same modeling of the relationship of various cellular responses with drug-induced adduct levels was performed for the widely used and well-characterized glioblastoma cell line LN229 (MGMT lacking, p53 functionally wt). The $O^\delta$MeG adduct levels (calculated from the dose-response curve in Fig. 1) were related to DSBs, apoptosis and senescence values from our previous report [34] and supplementary data (Fig. S6). The data revealed that γH2AX foci (Fig. 6A), apoptosis (Fig. 6B) and senescence (Fig. 6C) increased linearly with the $O^\delta$MeG adduct level per cell, and no thresholds were apparent. In this case, an increase of apoptosis by 1% in the population was calculated to require 950 $O^\delta$MeG adducts per cell, and senescence 410 $O^\delta$MeG adducts per cell. Assuming a 1:1 correlation between γH2AX foci and DSBs, we calculated that 350 $O^\delta$MeG adducts are required for the induction of one DSB in LN229 cells. LN229 cells expressing MGMT were completely refractory to the induction of γH2AX foci, apoptosis and senescence (supplement Fig. S6).

Discussion

TMZ, front-line drug in the treatment of high-grade malignant glioma, is effective if the tumor lacks MGMT or expresses the repair protein at a low level, i.e. <30 fmol/mg protein [39]. These tumors are defined as “methylated” since MGMT promoter CpG methylation correlates with silencing of the gene [40] and, as a consequence, lack of or low MGMT protein expression and enzyme activity [17]. Although the curative effect is significant, the response is not dramatic and the prognosis of GBM is still bleak with a median survival of only 14.6 months (12.6 and 23.4 months in the MGMT-unmethylated and MGMT-methylated subgroups, respectively) [41]. Although recent phase III clinical trials revealed that the median overall survival for adult patients with newly diagnosed GBM can reached up to 20 months, indicating a trending increase in survival, the prognosis is still bad with 5-year overall survival rates of less than 10% [42]. Given the mechanism of action of TMZ and the low curative response, some questions arise which are in the literature controversially discussed [27, 30] and thus need to be addressed experimentally. 1) Do GBM cells really die (by apoptosis or other forms of cell death) following TMZ treatment or does the drug just exert a cytostatic effect? 2) Are the doses used for patient's treatment high enough to kill cancer cells in their body? This question implies that low doses are ineffective and that threshold doses do exist below which cytotoxicity does not occur. 3) How many $O^\delta$MeG ad-
ducts are required for eliciting a specific effect such as apoptosis, necrosis and senescence? Here, we present data on the amount of O\(^6\)MeG induced by TMZ in established GBM cell lines and set it in relation to the level of DSBs, apoptosis, necrosis, overall cell death and cellular senescence.

Endpoints triggered by O\(^6\)MeG

MGMT-lacking GBM cells respond to treatment with TMZ in the dose range up to 50 \(\mu\)M with the induction of apoptosis, autophagy and senescence. These effects were nearly completely vanished in isogenic MGMT expressing cells (Fig. S6), supporting that O\(^6\)MeG induced by TMZ is responsible for these endpoints [8]. Since autophagy is a survival pathway and cellular senescence a cytostatic effect, it is pertinent to conclude that O\(^6\)MeG triggers both survival and death pathways. The apoptosis pathways triggered by O\(^6\)MeG are well described [25, 27]; they involve activation of both the death receptor FAS/CD95/APO1 and the intrinsic mitochondrial pathway, dependent on the cellular background [43]. Recently we showed, using LN229 cells, that apoptosis is a linear function of dose [34], which was substantiated in the present work with A172 cells. Using hockey-stick modelling [38], we show that there is no threshold for TMZ/O\(^6\)MeG-induced apoptosis in LN229 cells, and a marginal threshold in A172 cells at a dose level of 2.5 \(\mu\)M. In MGMT expressing cells the threshold is clearly higher, dependent on the MGMT expression level. Previous work with isogenic cell strains expressing different MGMT levels showed that the protection caused by MGMT is a linear function of molecules per cell [4]. Comparing the different endpoints triggered by O\(^6\)MeG quantitatively, it is interesting to note that the yield of cell death by apoptosis (and necrosis, which is a minor trait) is significantly lower than senescence. Thus, we measured 120 h after TMZ treatment in LN229 cells 25.4% apoptosis and 48.8% senescence, and in A172 cells 7.5% apoptosis and 33.3% senescence. Similar to apoptosis, senescence increases linearly with dose, without a no-effect threshold. Overall, O\(^6\)MeG elicits both a cytotoxic and cytostatic effect by activating, in the same dose range, the apoptosis and the senescence pathway; the latter is the predominant trait. This occurs already at low dose levels (<50 \(\mu\)M), which can be achieved in vivo in a therapeutic setting [44].

Is TMZ effective in killing cancer cells in vivo?

Treatment of patients with TMZ occurs daily by different schedules [2, 29, 45, 46]. The serum half-life of TMZ is about 2 h and the serum concentration of TMZ has been determined to be in the range of 20 up to 70 \(\mu\)M [1, 47-51]. Thus, in a therapeutic setting with a single oral dose of 150 mg/m\(^2\), the peak plasma concentration was, on average, 28.4 \(\mu\)M (5.5 \(\mu\)g/ml) and the brain interstitium concentration was 1.5 \(\mu\)M (0.3 \(\mu\)g/ml) [52]. In another study, following oral 200 mg/m\(^2\)
TMZ, the plasma peak level was 72 µM and the concentration in the cerebrospinal fluid was 9.9 µM [53]. Overall, the TMZ concentration at the target organ seems to be rather low (1-10 µM), which may lead to the conclusion that the TMZ alkylation level is not high enough to exert a cytotoxic effect on GBM cells. Given the fact that p53 plays a supporting role in apoptosis and senescence triggered by O6MeG [31, 37], the supposition is nourished by the assumption that DNA damage activated p53 pathways involving p53ser15 and HIPK2/p53ser46 play a protective role at a low, and a proapoptotic role at a high genotoxin level, respectively [54]. This model implies that thresholds exist that regulate the balance between life and death [44]. Therefore, we anticipated that cell death is not induced at low DNA damage levels. The supposition that TMZ is ineffective in glioma treatment is further fueled by the supposition that TMZ exerts only cytostatic, but not cytotoxic activity [30].

Here, using the well-characterized glioblastoma cell lines A172 and LN229, we show that the amount of O6MeG in the DNA and the number of DSBs increase linearly with dose of TMZ, and the same was observed for apoptosis and senescence. We did not observe a threshold for DSBs (as measured by γH2AX and 53BP1 foci formation), apoptosis, necrosis and senescence (see also our previous report [34]). This finding has implications regarding underlying mechanisms. It indicates that upon DDR activation by O6MeG-triggered secondary lesions (i.e. MMR-mediated gaps and DSBs resulting from them during replication) cells make a decision between survival and death in a stochastic manner and, at least in this cell system, there does not appear to be a threshold dose below which cell death pathways are not activated. Interestingly, at all dose levels we observed the induction of apoptosis and senescence, indicating that in the cell population cytotoxic and cytostatic effects are brought about at the same time by the same critical lesion O6MeG, although to a different extent. If we translate these findings to the tumor in vivo, we can posit that even at a low concentration TMZ elicits a cytotoxic (apoptosis and at very low degree necrosis) and a cytostatic (senescence) effect. It should also be considered the possibility that repeated low-dose treatment leads to an accumulation of O6MeG in MGMT-deficient tumor cells and an amelioration of the cytotoxic effects. Overall, the findings strongly support the view that repetitive treatments with low doses of TMZ (according to the metronomic schedule) are effective and represent a reasonable treatment strategy.

How many O6MeG adducts are required for inducing DSBs, p53ser15, apoptosis, necrosis, and senescence?

Here, we have quantified for the first time O6MeG adducts induced by TMZ in glioblastoma cells and set it in relation to the amount of DSBs, p53ser15, apoptosis, necrosis, and cellular senescence. From the corresponding dose-response curves we calculated the amount of O6MeG ad-
ducts that are required for eliciting a specific effect. The results are compiled in Table 1. The data show that 48 and 21 $O^6$MeG/10^7 nt give rise to 1 DSB (measured by a $\gamma$H2AX focus) in A172 and LN229 cells, respectively. This corresponds to 490 and 350 $O^6$MeG per cell that result in a DSB in A172 and LN229, respectively. The formation of one 53BP1 focus needs 1,460 $O^6$MeG, and colocalized foci were observed at a level of 3,350 $O^6$MeG in A172 cells. A twofold increase of phosphorylated p53ser15 needed 4,700 $O^6$MeG, a twofold increase in p21 4,830 $O^6$MeG, and an increase in apoptosis by one percent required an adduct level of 2,560 and 950 $O^6$MeG in A172 and LN229, respectively. Necrosis was measured only in A172, and an increase by 1% was seen at 5,220 $O^6$MeG. Overall, for inducing 1% cell death (apoptosis and necrosis) about 1,580 $O^6$MeG adducts are needed. Senescence is triggered already by lower amounts of $O^6$MeG, with 440 and 410 $O^6$MeG adducts needed for increasing the senescence level by 1% in A172 and LN229 cells, respectively.

In Table 1, we also show the effect level brought about by a dose of 20 µM TMZ, which induces 14,000 and 20,600 adducts per cell in A172 and LN229, respectively. This dose induced 32 DSBs ($\gamma$H2AX foci) per A172 cell, a 3-fold increase of p53ser15 (which was saturated already with a dose of 15 µM TMZ), a 3-fold increase in p21 (saturated at a dose level of 10 µM TMZ).

Under these conditions, 12.3% of cells were dead and 35.4% of cells showed the senescence phenotype as measured 120 h after the onset of treatment. Overall, this low dose caused significant genotoxic and cytotoxic effects. It should be noted that long-term cultivation of TMZ-treated cells resulted in cell loss in the subsequent passages, which is explained by $O^6$MeG triggered cell death in >2 post-treatment cell cycles, as demonstrated previously [10].

$O^6$MeG is a most interesting DNA damage as it is not only cytotoxic, but also able to trigger genotoxic end points such as sister chromatid-exchanges (SCEs), point mutations, chromosomal aberrations and DNA single- and double-strand breaks. In previous work, it was shown that O6-methylating agents require 2 cell cycles for SCEs to be formed, indicating secondary lesions to be involved [55]. This was confirmed using MGMT-isogenic cell lines, demonstrating that critical events are happening in the post-treatment cell cycle [56]. The conversion probability for $O^6$MeG into SCEs was determined in two independent studies: In MGMT lacking CHO-9 cells it was shown that 30 $O^6$MeG per cell give rise to 1 SCE [57] while for a glioblastoma cell line the relationship was 43 $O^6$MeG/SCE [58]. This is a very high conversion rate compared to other DNA lesions, e.g. UV-induced pyrimidine dimers with 600 $O^6$MeG/SCE [59]. Regarding cytotoxicity, from the slope of survival curves (colony formation) it was estimated that 6,650 $O^6$MeG induce one lethal hit [58]. It is important to note that colony formation, which is frequently used as a highly sensitive indicator of cytotoxicity, is biased by the fact that non-toxic events such as cell
cycle inhibition and induced senescence reduce the size and number of colonies and therefore simulate cytotoxicity. For this reason, survival curves on the basis of colony formation can hardly be compared with dose-response curves based on apoptosis and necrosis, which are clear cell death indicators. Although the amount of $O^6$MeG induced in A172 and LN229 cells were rather similar, the amount of DSBs and the levels of apoptosis and induced senescence were not identical, which is expected in view of the complex pathways involved, including the conversion of $O^6$MeG into critical secondary lesions, triggering the DDR and activation of survival and death pathways.

**Conclusions**

$O^6$MeG is a highly recombinogenic, mutagenic, genotoxic, autophagy, senescence and apoptosis-inducing lesion [4, 5, 8, 25]. It triggers with high efficiency SCE formation, which are cytogenetic events resulting from a damage tolerance process [60], followed by DSBs, senescence and apoptosis. The linear dose-effect relationship for $O^6$MeG and DSBs strongly indicates that in the therapeutic dose range, glioblastoma cells in vivo are damaged, causing responses that include cell death and cellular senescence, which appears to be the major trait. Since the $O^6$MeG/MMR/DSB-triggered downstream survival and death pathways of senescence and apoptosis are activated at the same time in the cell population, complete elimination of cancer cells cannot be expected by this therapeutic strategy. The possibility of senescent cells being reactivated to go back into cycle and start to proliferate must also be considered. Furthermore, TMZ-induced senescent glioblastoma cells secrete proinflammatory cytokines [26], which may drive tumorigenesis. Therefore, the elimination of senescent cells by senolytic drugs should be a further goal to improve therapeutic efficacy, as opposed to purely increasing the dose of TMZ in target tumor tissue. Finally, it is reasonable to postulate that quantitative adduct formation could serve as a potential biomarker of response and/or a determinant of adequate TMZ intra-tumoral dose. Therefore, molecular dosimetry of $O^6$MeG in relation to cellular effects is important for elucidating critical damage levels in cancer and normal tissues during and after therapy.

**Supplementary Materials:** Supplementary materials can be found at....

**Disclosure of potential conflicts of interest:**

No potential conflicts of interest were disclosed.

**Author Contributions:**
Conception and design: BK
Experimental design and development of methodology: BS, SG, LB, SS, BK
Acquisition of data: BS, SG, YH, LB
Analysis and interpretation of data: BS, SG, BK
Figures and statistics: BS, LB, BK
Writing of manuscript: BK
Review and revision of manuscript: BS, SG, LB, YH, SS, BK
Administrative, technical, or material support, funding: SS and BK
Study supervision: BK

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Kaina B. Mechanisms and consequences of methylating agent-induced SCEs and chromosomal aberrations: a long road traveled and still a far way to go. *Cytogenet Genome Res* 2004; 104: 77-86.

Table 1
DSBs represented by γH2AX and 53BP1 foci, cell death and cellular senescence induced in A172 and LN229 cells upon treatment with TMZ dependent of $O^6$MeG adducts per cell. Data are from figures 6, 7 and 8. nd, not determined.
<table>
<thead>
<tr>
<th>Effect</th>
<th>A172</th>
<th>LN229</th>
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</thead>
<tbody>
<tr>
<td>γH2AX</td>
<td>490 per focus</td>
<td>32 foci</td>
</tr>
<tr>
<td>53BP1</td>
<td>1,460 per focus</td>
<td>13 foci</td>
</tr>
<tr>
<td>Colocalization</td>
<td>3,350 per focus</td>
<td>4 foci</td>
</tr>
<tr>
<td>p53ser15</td>
<td>4,700 per 2-fold</td>
<td>3-fold(^1)</td>
</tr>
<tr>
<td>p21</td>
<td>4,830 per 2-fold</td>
<td>3-fold(^2)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>2,560 per 1% increase</td>
<td>7.7%</td>
</tr>
<tr>
<td>Necrosis</td>
<td>5,220 per 1% increase</td>
<td>3.6%</td>
</tr>
<tr>
<td>Total cell death</td>
<td>1,580 per 1% increase</td>
<td>12.3%</td>
</tr>
<tr>
<td>Senescence</td>
<td>440 per 1% increase</td>
<td>35.4%</td>
</tr>
</tbody>
</table>

\(^1\) saturated at 10,500 O\(^6\)MeG lesions; \(^2\) saturated at 7,000 O\(^6\)MeG lesions
Figure Legends

Fig. 1
Amount of \( O^6 \)MeG induced in glioblastoma cells (LN229) upon treatment with TMZ. (A) Adducts induced after 2, 3, 4 and 24 h exposure of LN229 cells with 50 µM TMZ. There is no significant difference between the levels. (B) Amount of adducts induced in LN229 and LN229-MGMT cells exposed for 3 and 24 h to 50 µM TMZ. Data are the mean of three independent determinations ±SEM, p<0.0001. (C) \( O^6 \)MeG in glioblastoma cells exposed to TMZ for 3 h in A172 cells and (D) in LN229 and LN229-MGMT cells. Data are the mean of three independent determinations ±SEM. A172: y=0.6873*x; LN229: y=0.6239*x; LN229-MGMT: y=0.1569*x-2.151

Fig. 2
Amount of γH2AX and 53BP1 foci induced in A172 glioblastoma cells upon treatment with increasing doses of TMZ. Cells were fixed and immunostained 72 h after the addition of TMZ to the medium. (A) Images from representative examples. (B) Dose-response for γH2AX, (C) 53BP1 and (D) colocalized foci. Data are the mean of four independent determinations ±SEM. The following equations describe the curves for γH2AX y=1.416*x+3.431, for 53BP1 y=0.4781*x+3.817, for colocalized foci y=0.2089*x+0.2138.

Fig. 3
Amount of p53, p53ser15 and p21 in A172 cells treated with various doses of temozolomide. (A) Exponentially growing cells were harvested 120 h after treatment with TMZ. HSP90 was used as loading control. A representative Western blot is shown. (B) Quantification of p53, (C) p53ser15 and (D) p21 in A172 cells treated with TMZ. Data are the mean of 3 independent experiments ±SEM. Dose-response for (E) p53ser15 and (F) p21 in A172 cells at the low TMZ dose range (up to 15 µM). The curves do not indicate a threshold dose.

Fig. 4
Time-course of apoptosis (A), necrosis (B), total cell death (C) and senescence (D) after treatment with 50 µM TMZ of A172. Data are the mean of at least 3 independent experiments ±SEM. * p<0.05, ** p<0.01. Dose-response of (E) apoptosis, (F) necrosis and (G) total cell death in A172 cells treated with TMZ and measured 120 h after the start of treatment. Data are the mean of 5 independent experiments measured in duplicate ±SEM. (H) Cellular senescence (C12FDG+) induced by TMZ in A172 cells. Data are the mean of 4 independent experiments ±SEM.

Fig. 5
Cellular responses as a function of \( O^6 \)MeG induced by TMZ in A172 glioblastoma cells. (A) γH2AX; (B) 53BP1; (C) colocalized foci; (D) apoptosis; (E) necrosis; (F) total cell death; (G) senescence as a function of the \( O^6 \)MeG content per cell. Data are from Fig. 1-4.

Fig. 6
Responses induced in LN229 cells by TMZ in dependence on the amount of \( O^6 \)MeG per cell: (A) γH2AX, (B) apoptosis, and (C) senescent cells in the population following treatment with increas-
ing concentrations of TMZ. Data are from Fig. 1, He and Kaina 2019 [34] and supplementary material.
Figure 1
Figure 2

**A**

<table>
<thead>
<tr>
<th>γH2AX</th>
<th>53BP1</th>
<th>Colocalized</th>
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<td>Control</td>
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</tbody>
</table>

**B**

\[
y = 1.416 \times x + 3.431 \\
p < 0.001
\]

**C**

\[
y = 0.4781 \times x + 3.817 \\
p < 0.001
\]

**D**

\[
y = 0.2089 \times x + 0.2138 \\
p < 0.001
\]
Figure 3
Figure 4
Figure 5
Figure 6
Molecular Cancer Therapeutics

Molecular dosimetry of temozolomide: Quantification of critical lesions, correlation to cell death responses and threshold doses

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