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# Chapter 6

## **Anti-tumour effects by a trimodal combination of temozolomide, meloxicam and X-rays in cultures of human glioma cells**

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**Abstract**

*Purpose:* To investigate the possible cytotoxic interactions between the chemotherapeutic drug temozolomide (TMZ) and the cyclooxygenase-2 inhibitor meloxicam (MLC) or of both drugs combined with X-rays in three human glioma cell lines (D384, Hs 683 and U251).

*Materials and methods:* Cells were exposed to TMZ (96 hours) and MLC was co-incubated during the last 24 h. Thereafter, cells were irradiated with X-rays and plated for a clonogenic assay. Total cell numbers and the numbers of surviving cells were determined to study the recovery of the cell populations (up until 19 days) following different combinations of TMZ, MLC and X-rays.

*Results:* The combination of MLC and TMZ caused an enhanced cytotoxic effect in D384 and Hs 683. Various treatment combinations demonstrated significant radiation enhancement in all three cell lines. Long-term observations of D384 cells demonstrated that the repopulation rates of the surviving cells are far less affected by the various treatment protocols than those from the non-surviving cells.

*Conclusions:* The present study demonstrates that a combination of TMZ and MLC resulted in a significant potentiation of their cytotoxicity in D384 and Hs683. The combination of these two drugs can also cause considerable enhancement of the radiation response in human glioma cell lines, although only D384 cells benefit from trimodal over bimodal treatment.

## Introduction

The inducible cyclooxygenase-2 (COX-2) protein plays a role in pathological conditions and cancer and catalyses the rate-limiting step in prostanoid synthesis by converting arachidonic acid into prostaglandin H<sub>2</sub>. Specific cyclooxygenase-2 inhibitors like meloxicam (MLC) have demonstrated anti-tumour properties both *in vitro* and *in vivo* [13, 17, 21, 40, 41]. Actions accomplished by COX-2 inhibitors include inhibition of cell proliferation and angiogenesis, as well as induction of apoptosis.

Overexpression of the COX-2 protein has been found in many tumour types including gliomas. The expression of COX-2 in gliomas is associated with a higher malignancy of tumours and shorter survival times [9, 14, 24, 30]. Tumours of glioblastoma multiforme (GBM) patients often contain a high level of COX-2 protein. Therefore COX-2 may be a potential therapeutic target for the treatment of these tumours.

Temozolomide (TMZ) is an alkylating agent that can add methyl groups to the O<sup>6</sup> position of guanine (O<sup>6</sup>-methylguanine or O<sup>6</sup>-MeG) by its intermediate 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). Incorporation of O<sup>6</sup>-MeG adducts into the DNA can result in futile repair attempts by the mismatch repair system; leading to base/base mismatches and eventually cell death [5, 12, 15]. The DNA repair protein O<sup>6</sup>-methylguanine-DNA-methyl-transferase (MGMT) is able to remove the methyl group from the O<sup>6</sup>-position of guanine, thereby counteracting the DNA damage caused by TMZ [3, 7]. Repair by MGMT is an important mechanism of resistance to TMZ.

Since 2005, TMZ has been qualified for patients with newly diagnosed GBM as an additional therapy next to surgery and radiation therapy [33]. Patients showing (hyper)methylation of the MGMT gene promoter (causing transcriptional silencing) had prolonged survival after treatment with radiotherapy and TMZ, as compared to patients treated with radiotherapy alone [10, 11]. As a result prognosis of GBM patients has improved and the median survival time is increased to 14 months.

Several studies in various tumour types have shown that the effects caused by cytotoxic drugs may be enhanced by COX-2 inhibitors [20, 22, 32]. Furthermore, combinations of  $\gamma$ -radiation and the COX-2 inhibitors celecoxib, MLC or NS-398 were investigated previously in our lab [2, 19]. The COX-2 inhibitor MLC demonstrated to be the most potent radiosensitiser of the tested drugs and was therefore the first choice for further (radiation) studies. MLC has the advantage that it can be used at relative high concentrations, thereby being cytostatic without causing significant cytotoxicity [2]. On the other hand, the chemotherapeutic drug TMZ has proven its value in GBM therapy. Both MLC and TMZ were shown to be potent radiosensitisers in cultures of human glioma cells [2, 4, 18, 35, 38]. An additional study with both drugs in combination with ionising radiation is therefore warranted.

In the present study we investigated the cell kinetics, i.e. cell survival, cell growth and cell cycle analysis, of three human glioma cell lines in response to single or combined treatments with TMZ, MLC and X-ray irradiation.

## **Methods**

### *Cell lines and culture*

Three established glioma cell lines were used: D384 (astrocytoma grade III; [1]), Hs 683 (GBM; [23]), and U251 (GBM; [26]). The cell lines were kindly provided by Dr. C.H. Langeveld, (Dept. Neurology, VU University Medical Center, Amsterdam, The Netherlands). Cell lines were cultured at 37°C in (Leibovitch) L15 medium with 10% fetal calf serum (Gibco Life Technologies, Breda, The Netherlands) and in addition 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen, Breda, The Netherlands).

### *Experimental procedures*

Experiments were conducted on exponentially growing cells that were seeded three days before the commencement of treatments. MLC (Boehringer Ingelheim, Ingelheim, Germany) and TMZ (Schering-Plough RS, Utrecht, the Netherlands) were prepared freshly and dissolved in dimethyl sulfoxide (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) before dilution in culture medium. Final concentrations of the solvent did not affect cell proliferation or clonogenic cell survival. The 24-h incubation period for MLC and the 4 times 24 h repeated treatment interval for TMZ were chosen according to previous studies [2, 38]. For combinations of TMZ and MLC, MLC was added at 72 h. Following treatment with drugs, cells were irradiated with single doses of X-rays (1-6 Gy) in their continued presence. Irradiation was executed at room temperature with 80-kV orthovolt X-rays (Pantak Therapax SXT 150) at a dose rate of 1 Gy per min. Cell survival was assessed by the clonogenic capacity of single cells. Experiments were repeated at least twice.

### *Clonogenic assay*

Immediately following the experimental treatments, cells were dispersed with trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco Life Technologies) and plated in adequate numbers. The amount of seeded cells (500 - 50000 per 25 cm<sup>2</sup>) depended on the estimated survival. After an incubation of 10 days, the developed cell colonies were fixed with 100% ethanol and stained with 5% Giemsa solution (Merck, Darmstadt, Germany). Colonies containing 50 cells or more were counted and considered as cells with an unaffected clonogenic capacity. Average plating efficiencies for D384, Hs 683 and U251 cells were 68%, 31% and 38% respectively.

Survival (S) data after dose (D) of radiation are presented after correction for the plating efficiencies of relevant control cells as surviving fractions (SF= S(D)/S(0)). So, raw data for each individual survival curve were normalised for the survival of the associated preirradiation treatment, presenting the effect of radiation treatment alone.

### *Statistical analysis*

Single-dose radiation survival curves were constructed according to the linear-quadratic (LQ) formula  $S(D)/S(0)=\exp(-\alpha D+\beta D^2)$  using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Radiation survival curves were analysed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) statistical software as described by Franken et al. [6]. Cell survival curves of different treatment groups were considered statistically different when  $p < 0.05$ .

### *Cell cycle analysis*

The experiment was performed as described previously [2, 19]. Fluorescence-activated cell sorter (FACS) samples were prepared after treatment with 96 h TMZ (0-2-4-6-8-10  $\mu\text{M}$ ) or 24 h MLC (0-250-500-750  $\mu\text{M}$ ). Data for 0 and 750  $\mu\text{M}$  MLC in D384 and U251 cells were already published before [2, 19].

### *Repopulation assay*

The repopulation assay combines both cell proliferation and clonogenic cell death. Cell proliferation curves were created to study the recovery of D384 cells after the experimental treatments. Cell numbers (including both surviving and ultimately non-surviving cells) and the clonogenic capacity were determined during the recovery until 7-19 days depending on the experimental condition. To establish the repopulation of the cultures with clonogenic cells, the cell proliferation and clonogenic survival data were combined to yield the surviving cell population [35, 37]. The population doubling times (Td) were calculated with data obtained from the graphs.

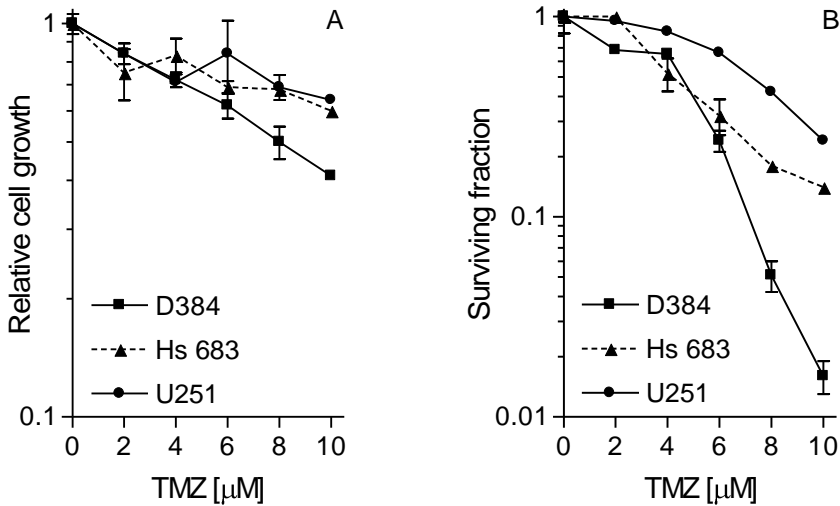
## **Results**

### *Effect of TMZ on glioma cell kinetics*

Glioma cells were exposed to various doses of TMZ. After 96 h of incubation cells were counted and plated for determination of the clonogenic capacity. The daily exposure with TMZ for 96 h was found to give the best results with respect to enhancement of cytotoxicity as opposed to shorter incubation times of 24, 48 or 72 h [38].

Figure 1A shows the relative cell growth of the three cell lines after 96 h for various concentrations of TMZ. TMZ caused a concentration dependent inhibition of cell growth in D384. Hs 683 and U251 cells were hardly affected.

Increasing concentrations of TMZ also correspond with an increasing loss of clonogenic capacity (Figure 1B). D384 cells show the highest response whereas U251 is the most resistant cell line.



**Figure 1.** Effect of TMZ on glioma cell kinetics in the cell lines D384 (black square), Hs 683 (black triangle), and U251 (black circle). (A) Relative cell growth after 96 h of incubation with TMZ. (B) Clonogenic cell survival after 96 h of TMZ exposure. Survival data are corrected for the plating efficiency of untreated control cells leading to normalised data. Typical experiments are shown. Error bars indicate the standard deviation for duplicates.

Cell cycle analysis was performed under the same conditions. The results are shown in Table 1. D384 and U251 cells were accumulated in the G2/M phase of the cell cycle, whereas no clear changes were seen for Hs 683 cells.

**Table 1.** Cell cycle distribution of D384, Hs 683 and U251 cells after 96 h exposure to 0, 2, 4, 6, 8 or 10 μM TMZ.

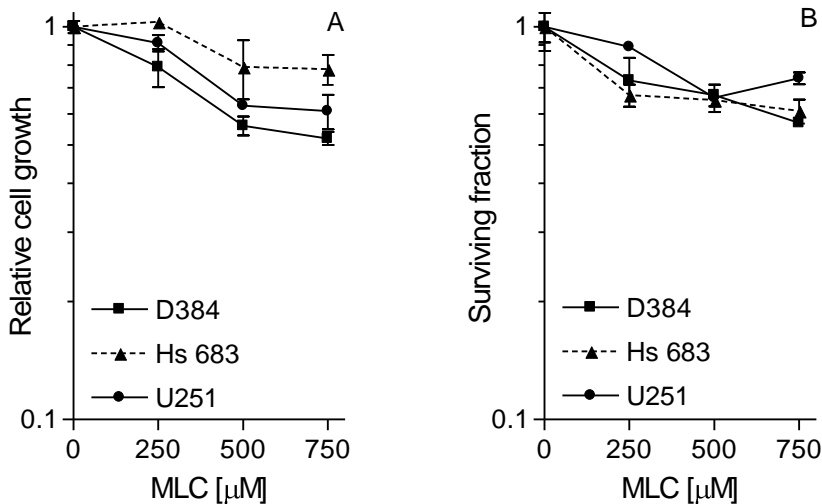
Cell line	Cell cycle phase (%)	TMZ dose [μM]					
		0	2	4	6	8	10
D384	G1	60	54	48	29	23	18
	S	26	22	21	32	32	44
	G2/M	13	23	32	39	46	38
Hs 683	G1	53	54	48	46	42	44
	S	29	8	18	28	30	27
	G2/M	18	38	34	26	29	29
U251	G1	49	34	30	29	23	22
	S	34	31	32	29	33	32
	G2/M	17	34	37	42	44	46

### Effect of MLC on glioma cell kinetics

Glioma cells were exposed to various doses of MLC. After 24 h of incubation cells were counted and plated for determination of the clonogenic capacity. Incubation times with MLC beyond 24 h did not further change the already severe cytostatic effects on cell proliferation [2].

Figure 2A shows the relative cell proliferation of the three cell lines after 24 h exposure to MLC. Growth inhibition was strong from a concentration of 500  $\mu\text{M}$  and occurred in all three cell lines. At the highest concentration of 750  $\mu\text{M}$  MLC it even resulted in a full arrest of cell proliferation in D384 and U251 cells.

In contrast, the clonogenic capacity of these three cell lines was only moderately affected by MLC treatment (Figure 2B). At 750  $\mu\text{M}$  MLC loss of clonogenic capacity was limited to 50 % after 24 h at most. Therefore this concentration was used throughout for the trimodal combination experiments.



**Figure 2.** Effect of MLC on glioma cell kinetics in the cell lines D384 (black square), Hs 683 (black triangle), and U251 (black circle). (A) Relative cell growth after 24 h of incubation with MLC. (B) Clonogenic cell survival after 24 h of MLC exposure. Survival data are corrected for the plating efficiency of untreated control cells leading to normalised data. Typical experiments are shown. Error bars indicate the standard deviation for duplicates.

The data from the cell cycle analysis are shown in Table 2. Accumulation in the G2/M phase of the cell cycle was seen for D384 and U251 cells. Hs 683 cells, on the other hand, accumulated in the G1 phase after treatment with MLC (Table 2).



**Table 2.** Cell cycle distribution of D384, Hs 683 and U251 cells after 24h exposure to 0, 250, 500 or 750  $\mu\text{M}$  MLC.

Cell line	Cell cycle phase (%)	MLC dose [ $\mu\text{M}$ ]			
		0	250	500	750
D384	G1	51*	46	38	40*
	S	31*	22	33	33*
	G2/M	18*	32	29	27*
Hs 683	G1	45	52	61	60
	S	34	35	23	18
	G2/M	20	13	16	22
U251	G1	47*	51	44	41*
	S	36*	26	18	17*
	G2/M	17*	23	39	41*

\* Published before [2,19].

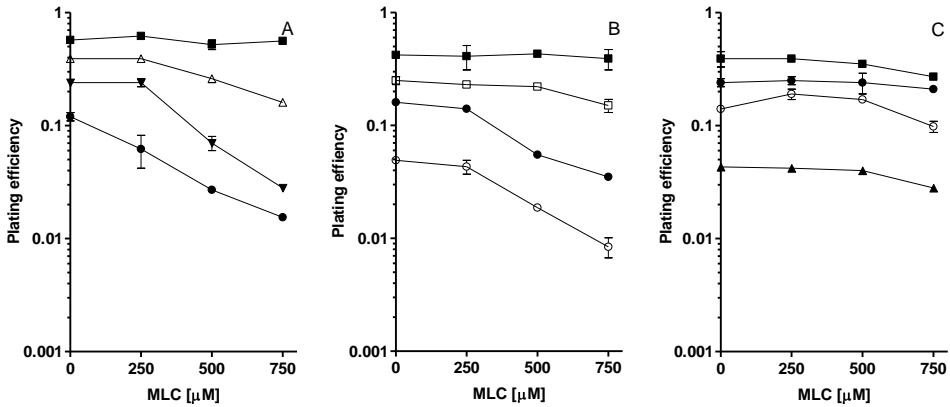
*Effect of combinations of MLC and TMZ on cell survival*

Before investigating the complex trimodal treatment including an irradiation with X-rays, the question was addressed how cell survival was affected by combinations of TMZ and MLC. Therefore the cells were exposed to various concentrations of the drugs, consisting of a repeated 24 h TMZ exposure during 96 h and MLC present during the last 24 h.

As mentioned before, MLC alone shows minimal cytotoxicity, but in the presence of increasing concentrations of TMZ, a significant enhancement of cytotoxicity is noticed in two of the cell lines. In D384 cells both 500 and 750  $\mu\text{M}$  MLC positively interacted with 2, 4, and 5  $\mu\text{M}$  TMZ (Figure 3A). A MLC concentration of 250  $\mu\text{M}$  only showed enhancement of the cytotoxic response after pre-treatment with 5  $\mu\text{M}$  TMZ. Hs 683 cells did display a positive cytotoxic interaction between 750  $\mu\text{M}$  MLC combined with all tested TMZ concentrations (Figure 3B). MLC concentrations of 500  $\mu\text{M}$  showed cytotoxic enhancement after 5 and 7.5  $\mu\text{M}$  TMZ, while no interaction after pre-treatment with TMZ was found at 250  $\mu\text{M}$  MLC. Any (positive) interaction between TMZ and MLC is absent in U251 cells (Figure 3C).

*Effect of combined drugs treatment and irradiation with X-rays*

For the trimodal combination experiments concentrations of TMZ were used that had similar effects on clonogenic cell death (isotoxic concentrations) as a single agent and also demonstrated an enhancement of cytotoxicity in combination with 750  $\mu\text{M}$  of MLC (Figure 3, except for U251). The selected concentrations of TMZ in D384 (2  $\mu\text{M}$ ), Hs 683 (2.5  $\mu\text{M}$ ) and U251 (5  $\mu\text{M}$ ) reduced the clonogenic capacity with approximately 37%.



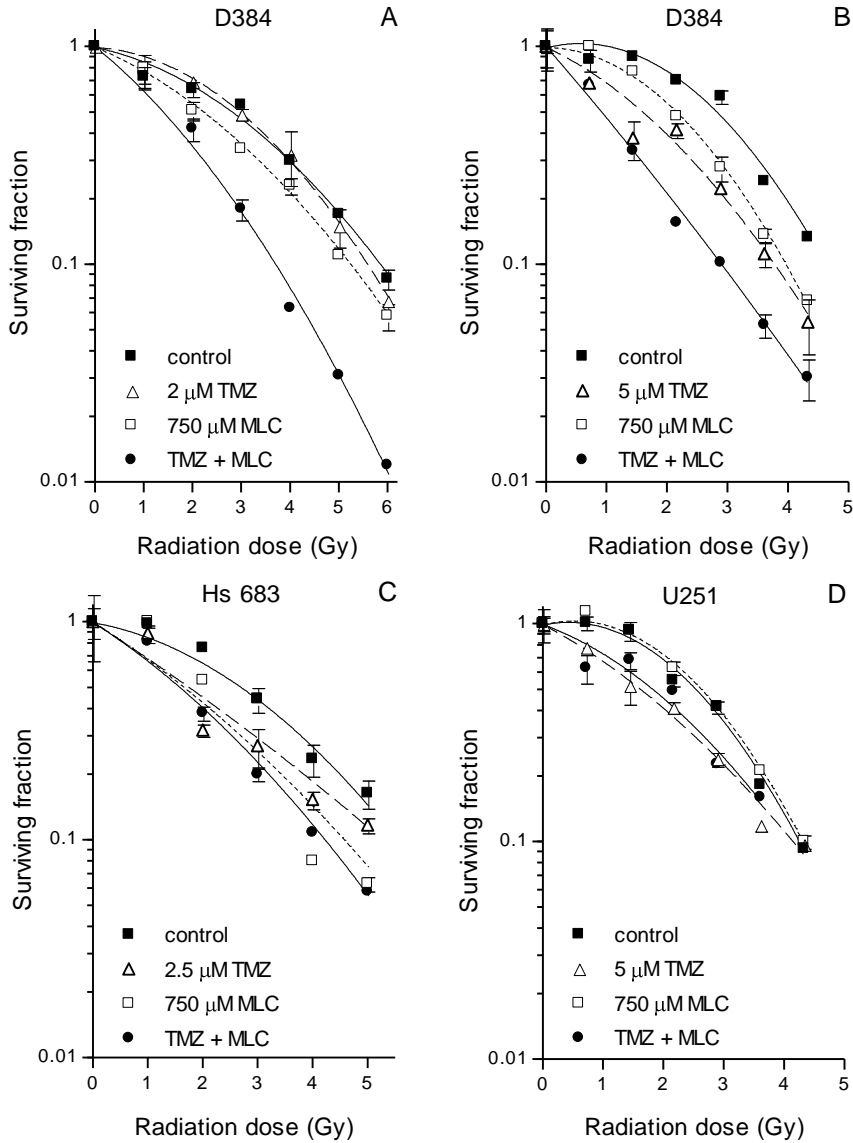
**Figure 3.** Effect of combinations of MLC and TMZ on clonogenic cell survival. D384 (A), Hs 683 (B), and U251 (C) cells were exposed to several doses of TMZ in the course of 0-96 h in combination with various doses of MLC (0-750  $\mu\text{M}$ ) during 72-96 h. Symbols represent: control (black square), 2  $\mu\text{M}$  TMZ (open triangle), 2.5  $\mu\text{M}$  TMZ (open square), 4  $\mu\text{M}$  TMZ (black diamond), 5  $\mu\text{M}$  TMZ (open circle), 7.5  $\mu\text{M}$  TMZ (black circle), and 10  $\mu\text{M}$  TMZ (black triangle). Typical experiments are shown. Error bars indicate the standard deviation for duplicates.

No significant radiation enhancement was found after treatment with TMZ as a single agent in D384 cells (n.s.,  $p = 0.431$ ) as demonstrated in Figure 4A. Both MLC alone and the combination of MLC with TMZ caused an enhancement of the radiation response ( $p < 0.05$ ), moreover the effect caused by the combination of both drugs on the radiation response was more effective than MLC alone ( $p < 0.05$ ).

D384 cells were also tested in more detail with a concentration of 5  $\mu\text{M}$  TMZ. A statistically significant enhancement of the radiation response was demonstrated by TMZ, MLC, or by the combination of both drugs as shown in Figure 4B (all  $p < 0.05$ ). Trimodal treatment with the combination of both drugs caused more radiation enhancement than treatment with either TMZ or MLC alone (both  $p < 0.05$ ).

The cell line Hs 683 also displays a statistically significant enhancement of the radiation response by TMZ, MLC, and the combination of drugs as presented in Figure 4C (all  $p < 0.05$ ). Although TMZ combined with MLC was more effective on the enhancement of the radiation response than TMZ as a single agent ( $p < 0.05$ ), it was not more effective than MLC alone (n.s.,  $p = 0.281$ ).

In contrast, U251 cells in Figure 4D exhibited no statistically significant effect of the radiation response by MLC as a single agent (n.s.,  $p = 0.298$ ). However, a statistically significant enhancement of the radiation response was found by either TMZ or the combination of TMZ with MLC (both  $p < 0.05$ ). Moreover, the radiation enhancement effect by TMZ alone was not different from the effect caused by the combination of TMZ and MLC (n.s.,  $p = 0.475$ ).

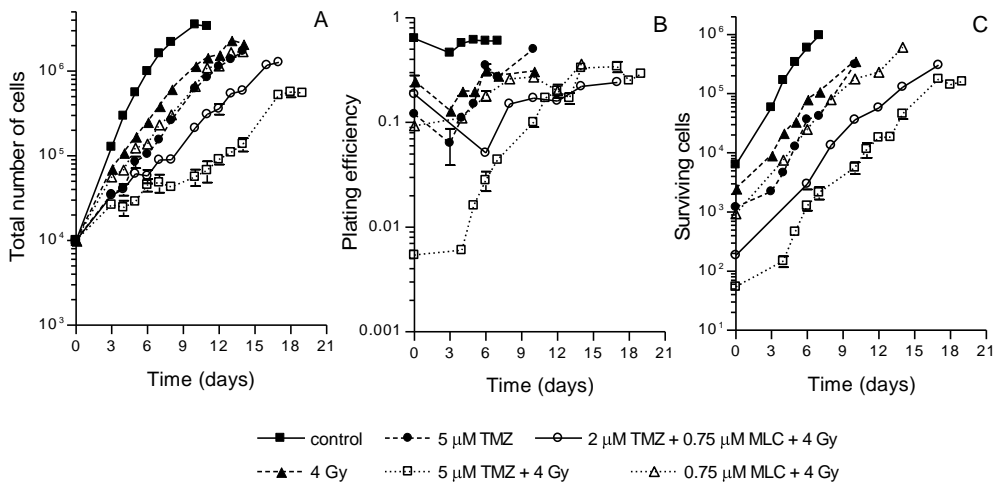


**Figure 4.** Effect of combined drugs treatment and irradiation with X-rays. Cells were exposed to 2 μM (D384, A), 5 μM (D384, B), 2.5 μM (Hs 683, C) or 5 μM (U251, D) TMZ for 96 h of which the last 24 h were co-incubated with 750 μM MLC prior to irradiation with X-rays (0-6 Gy). Symbols represent: control (black square), TMZ (open triangle), 750 μM MLC (open square), and TMZ + MLC (black circle). Survival data are corrected for the plating efficiency of relevant control cells (untreated, TMZ or MLC alone or TMZ and MLC combined) leading to normalised data. Typical experiments are shown. Error bars indicate the standard deviation for duplicates.

Enhancement of the radiation response is also demonstrated by the shape of the radiation survival curves. Treatment with the combination of both TMZ and MLC clearly reduced the shoulder of these curves in all three cell lines. Especially in the cell line D384 where the shoulder of the curve becomes even absent with trimodal treatment. This effect was also seen, although not always as prominent, in all three cell lines with TMZ and MLC as single agents, except for the survival curves with TMZ (2 $\mu$ M) in D384 cells and MLC in U251 cells, which are equal to the control curve with radiation alone.

### Repopulation assay

In addition to the clonogenic experiments, a repopulation assay was performed with D384 cells as a possible alternative for the assessment of cytotoxicity. Therefore D384 cells were treated with different combinations of drugs and X-rays in order to study the recovery of the cell population. A typical example of such an experiment is shown in Figure 5.



**Figure 5.** Repopulation assay. Cell numbers and clonogenic cell survival were determined following different treatments with drugs and/or X-rays in D384 cells. Symbols represent: control (black square), 4 Gy (black triangle), 5  $\mu$ M TMZ (black circle), 5  $\mu$ M TMZ + 4 Gy (open square), 750  $\mu$ M MLC + 4 Gy (open triangle), and 2  $\mu$ M TMZ + 750  $\mu$ M MLC + 4 Gy (open circle). A) Total number of cells, B) plating efficiency, and C) number of surviving cells (total number of cells x plating efficiency) during recovery after different treatments. Typical experiments are shown. Error bars indicate the standard deviation for duplicates.

Cell proliferation was monitored for at least 11 days following the various treatments as shown in Figure 5A. Simultaneously the clonogenic capacity of the cell populations was monitored, showing a time- and treatment-dependent recovery of the plating efficiency (Figure 5B). Combination of the data from both panels reveals the repopulation rates of the clonogenic cell populations as presented in Figure 5C, i.e.

the products of total cell growth (Figure 5A) and the fraction of clonogenic cells (Figure 5B). Table 3 shows that in case of the total cell population, the population doubling times (Td) for the various treatments (Figure 5A), varied strongly from 28 to 77 hours, while the Td values of the ultimately surviving cells (Figure 5C) are relatively unaffected (31 to 38 hours) by the different treatment protocols. Control cell populations showed no difference between total and surviving cell growth rates (Td = 24 h). Remarkably these results demonstrate that with respect to the repopulation rates, the ultimately surviving or clonogenic cells are far less affected by the various treatments than the non-surviving cells that dominate most of the treated cell populations in Figure 5A. Another conclusion from this experiment is that following treatments, the non-surviving cells remain present in significant amounts for a prolonged period of time and even may execute a number of cell divisions before they eventually die.

**Table 3.** Population doubling times (Td in hours) of the total cell population and for the surviving cell populations after different treatment protocols in D384 cells. Gy = Gray; TMZ = temozolomide; MLC = meloxicam

Treatment	Td (hours) total cell population	Td (hours) surviving cells
control	24	24
4 Gy	28	34
5 $\mu$ M TMZ	42	31
5 $\mu$ M TMZ + 4 Gy	77	38
750 $\mu$ M MLC + 4 Gy	34	31
750 $\mu$ M MLC + 2 $\mu$ M TMZ + 4 Gy	58	33

## Discussion

Even though the combination of fractionated radiotherapy with concomitant and adjuvant TMZ has significantly increased the median survival of patients with a primary GBM, both median and long-term survival are still limited [33]. In order to achieve further improvement, new additional therapy has to be developed. A potential group of new therapeutic agents are the COX-2 inhibitors, which have shown anti-tumour activity in various tumour types, including glioma.

In the present study we investigated the effects of the COX-2 inhibitor MLC in combination with TMZ or X-rays either single or both. The protocol that was followed (72 h TMZ followed by 24 h combined MLC and TMZ) revealed a clear enhancement of the cytotoxicity in two of the three cell lines (D384 and Hs 683).

The radiation enhancement properties of MLC and TMZ alone as shown in the literature [2, 4, 18, 35, 38] are confirmed by the present study for the glioma cell lines D384 (TMZ and MLC), Hs 683 (TMZ and MLC) and U251 (TMZ). An investigation of the radiosensitising potential for the combination of these drugs has not been reported so far. All three glioma cell lines tested, demonstrate radiation enhancement

by the combination of TMZ and MLC, although only in D384 cells the trimodal protocol was beneficial to both bimodal regimens.

Clinical studies on GBM patients combining radiotherapy, temozolomide and MLC have not been performed yet. However, a retrospective study was carried out [29] analysing the impact of different adjuvant chemotherapy regimens, including the COX-2 inhibitors rofecoxib and celecoxib, in a group of patients treated for a GBM compared to patients receiving only post-operative radiotherapy. The additional chemotherapy regimens consisted of high dose TMZ and low-dose TMZ combined with an inhibitor of COX-2. Both additional chemotherapy regimens had a longer median overall survival than patients receiving radiotherapy alone. Although the effect of this chemotherapy regimen (low-dose TMZ combined with an inhibitor of COX-2) did not exceed the effect of the regimen containing high dose TMZ.

A newly introduced approach to evaluate the results from chemo-radiation therapy in cell lines is the repopulation assay. This technique enables a detailed analysis of cell kinetics following treatments. This includes the monitoring of both cell proliferation and clonogenic cell survival during the recovery of the cell population for an extended period of time. By using this assay, long-term effects of various (combination) treatments (as reflected by the population doubling time in hours) can be ascertained for the total cell population, comprised of the surviving and the ultimately non-surviving cell populations. Since treatment effects may not only occur during the experimental treatment but also afterwards, results obtained from repopulation experiments as described in this study offer complementary information to the results obtained from clonogenic survival data.

Characteristic for a lethal event caused by ionising radiation is the delayed expression of cell death. Proliferation assays are therefore less feasible for studies with X-rays because of the extended survival with even a number of subsequent cell divisions of the ultimately non-clonogenic cells. This difference in cell kinetics after radiation is also demonstrated by the results of our repopulation experiments, where the population doubling time of the surviving cells, which develop detectable clones, was much less effected by the treatments than the proliferating but lethally affected cells, which ultimately turned into abortive clones. This again shows that the clonogenic assay is a more reliable parameter for assessment of treatment results than cell proliferation, unless cell proliferation assays are combined with clonogenic survival results to distinguish between the surviving and ultimately non-surviving cells.

The results with MLC for the cell lines D384 and Hs 683 either with TMZ alone or in combination with TMZ and X-rays are promising. However, the mechanisms by which MLC exerts its effects and interacts with TMZ and/or X-rays are unknown.

A possible explanation could be found within changes in the distribution of cells in the cell cycle. The G2/M phase is considered to be the most radiosensitive period of the cell cycle, because of the proximity and occurrence of mitosis. It is considered that DNA damage cannot be repaired in time and will then be fixed. The S phase (DNA replication) on the other hand, is the most radiation resistant part of the cell cycle.

Cell cycle analysis demonstrated that both D384 and U251 cells were accumulated in the G2/M phase of the cell cycle after incubations with either TMZ (96 h) or MLC (24 h). However no changes in cell cycle distribution were found in cell line Hs 683 after incubation with TMZ and incubation with MLC caused accumulation of cells in the G1 phase. Accumulation of cells in the G2/M phase thus seems to be consistent with the observed enhancement of the radiation response in both D384 and U251 cell lines after treatment with TMZ, however for the cell line Hs 683 this does not seem to be the case. In the case of MLC treatment, only the D384 cell line agrees with this cell cycle hypothesis. As the changes in cell cycle distribution showed no correlation with the specific responses to various treatments of these three cell lines, cell cycle redistribution probably does not explain the observed enhancement by combinations of drug and irradiation with X-rays in these cell lines.

Inhibition of sublethal damage repair (SLDR) has been reported in a few studies as a possible cause of the enhancement of the radiation response by COX-2 inhibitors [25, 27, 28]. SLDR could also be involved in radiosensitisation in this study, since pre-treatment with TMZ, MLC or the combination of both was able to reduce the shoulder of most curves, except with treatment of TMZ (2 $\mu$ M) in D384 cells and MLC in U251 cells. When using trimodal treatment in D384 cells the shoulder was even (completely) absent.

The DNA repair protein MGMT involved in TMZ resistance was undetectable in D384, Hs 683 and U251 cell lines as demonstrated in a recently published study by our group [36], which is in agreement with their high sensitivity for TMZ. Therefore, mechanisms of interaction between these two drugs and X-rays in these cell lines appear to be independent of TMZ-induced MGMT-mediated cellular resistance.

Since the cell lines D384 and U251 did not exhibit detectable levels of COX-2 protein expression as demonstrated by earlier studies from our lab [2, 19] and demonstrated different interaction results after the trimodality treatment, the mechanism(s) involved appear to be COX-2 independent. Several papers have reported about anti-tumour actions induced by specific COX-2 inhibitors by means of COX-2 independent mechanisms in various cancer cell lines of different origin and in tumour mouse models [8, 16, 21, 31, 34, 39].

Future research should therefore focus on further investigation of the mechanisms involved in the enhancement of combined drugs and radiation effects with TMZ and MLC and testing of this combination therapy protocol in vivo, before entering clinical trials. Another challenge will be to test more and different combinations of potential therapeutic agents with the standard therapy for GBM (TMZ and radiation). Multimodality treatment may well be the solution for further improving survival of GBM patients.

## **Conclusion**

The present study demonstrates that treatment of human glioma cells with a combination of TMZ and MLC can result in supra-additional cytotoxicity. Furthermore, combined MLC and TMZ treatment with X-rays can cause considerable

enhancement of the radiation response. This response can even be stronger than the radiation enhancement caused by treatment with MLC or TMZ alone.

### **Acknowledgements**

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