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ORIGINAL ARTICLE

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Combined effects of temozolomide and the ribonucleotide reductase inhibitors didox and trimidox in malignant brain tumor cells

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Abstract *Purpose*: Temozolomide (TMZ), an oral alkylating agent with good penetration of the blood-brain barrier, has shown efficacy in the treatment of malignant brain tumors. Ribonucleotide reductase (RR), the rate-limiting enzyme of DNA synthesis, seems to be a complementary target for combination chemotherapy of brain tumors. Trimidox (TX) and didox (DX) are two recently synthesized specific inhibitors of RR. The combinations of TMZ with TX or DX as a basis for synergistic chemotherapy protocols were tested in this study. *Methods*: The effects of the single drugs TMZ, DX, and TX, and the combinations TMZ/DX and TMZ/TX were evaluated in the human malignant glioma cell lines U87MG, T98G, LNZ308, and wt1119. In the latter, experiments were carried out in the presence or absence of wild-type p53 protein expressed under the control of a tetracycline-responsive transgene system. Cytotoxicity was evaluated by MTT assays. The isobologram and combination index (CI) method of Chou-Talalay were used to evaluate interactions between drugs. *Results*: All drugs demonstrated cytotoxicity in brain tumor cells. Synergistic cytotoxic effects ($CI < 1$) for TMZ and TX or DX at different dose levels were demonstrated in most of the examined cell lines. In some instances, however, drug combinations resulted in additive or even antagonistic effects. Toxicity of the single agents and synergy of the combinations did not correlate with wild-type p53 expression in the tumor cells. *Conclusion*: The tumor toxicity of TMZ as a single agent may be modified by combinations with the novel RR inhibitors DX and TX, and is synergistically enhanced in most cases. Depending on the combination ratio, the doses for each drug for a given degree of effect in the combination may be drastically reduced.

Keywords Brain tumor · Didox · Glioblastoma · Ribonucleotide reductase · Temozolomide · Trimidox

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Introduction

Brain tumors of glial origin (gliomas) are the most common primary tumors of the central nervous system (CNS) in adults [22]. Gliomas are exceptionally migratory and invade surrounding brain tissue early in their natural course [3, 17]. Malignant gliomas (WHO grades III and IV) are aggressive neoplasms with a uniformly fatal clinical course. Treatment of anaplastic astrocytoma (AA, WHO grade III) and glioblastoma (GBM, WHO grade IV) typically involves surgical resection or biopsy in combination with radiation and, in selected cases, adjuvant chemotherapy. However, even if gross total surgical resection is achieved and full adjuvant treatment is given, tumors generally recur within 1 year regardless of the initial response to treatment [3, 22].

An oral alkylating agent, temozolomide (TMZ, 3,4-dihydro-3-methyl-4-oxoimidazo-[5,1-d]-*as*-tetrazine-8-carboxamide), has recently been introduced into clinical practice for treatment of primary or recurrent malignant glioma, and has been shown to yield objective responses in more than 50% of patients with primary or recurrent GBM [2, 19]. TMZ has an excellent oral bioavailability and good penetration of the blood-brain barrier [10].

Resistance to TMZ in malignant brain tumor cells may occur relatively often and reduces the rate and durability of the tumor response.

Biosynthesis of deoxyribonucleotides (dNTP) from ribonucleotides is a crucial step of DNA synthesis and cell replication. As high concentrations of dNTPs are required for DNA synthesis, the activity of the key enzyme ribonucleotide reductase (RR) is closely related to the proliferative state of the cell. A tight correlation between RR activity and tumor growth rate has been demonstrated for several cancers [12, 13, 24]. Therefore, the enzyme is considered to be a promising target for cancer chemotherapy. Trimidox (TX, 3,4,5-trihydroxy-benzohydroxamid oxime) and didox (DX, 3,4-dihydroxy-benzohydroxamic acid), two recently synthesized RR inhibitors, are derivatives of polyhydroxy-substituted benzohydroxamates. DX has been investigated for its anticancer activity in a number of animal tumor models and also in phase I and II clinical trials [21]. TX is a DX analogue and still at the preclinical evaluation level [20]. TX and DX inhibit RR mainly by their capacity for scavenging free radicals. A further RR-inhibiting mechanism of these substances is iron deprivation, as iron is a cofactor of the R2-subunit of RR needed for generation and stabilization of free tyrosyl radicals [23].

In this study, the cytotoxicity and interactions of the combinations of TMZ with DX or TX were investigated in human malignant glioma cells. The study represented an attempt to improve the effects of monotherapy of glioma with TMZ, and to achieve drug reduction while retaining the same degree of antitumor efficacy. Also the influence of wild-type p53 on the effects of single drugs and their combinations was evaluated.

Materials and methods

Cell culture

The human malignant glioma cell lines U87MG (wild-type p53 protein) and T98G (mutant p53 protein) were obtained from ATCC (Rockville, Md.). LNZ308 (homozygous deletion of the *p53* gene) and the LNZ308-derived p53-inducible cell line wt1119 were a kind gift from Dr. E. van Meir, University of Lausanne, Switzerland [26]. In the wt1119 cell line, a wild-type human *p53* transgene (wt*p53*) was placed under the control of a *tet*-responsive element (TRE, Tet-off System; Clontech, Palo Alto, Calif.). Expression of the fully functional wt*p53* transgene can be suppressed by addition of doxycycline to the culture medium [26]. TRE-mediated regulation of wt*p53* transgene expression was verified by immunofluorescence staining with the monoclonal anti-p53 antibody DO7 (DAKO, Glostrup, Denmark).

Cytotoxicity assay

Tumor cells were seeded at a density of 4×10^3 cells per well in 96-well microtiter plates, and 24 h later the medium was supplemented with DX, TX, TMZ or combinations of TMZ with DX or TX. Both DX and TX were used at concentrations in the range 50 to 800 μM. TMZ was used at concentrations in the range 200 to 3200 μM. For the combination of TMZ/DX and TMZ/TX, concentrations of $100+25$, $200+50$, $400+100$, $800+200$, and $1600+400$ μM were used. After 4 days in culture, MTT (Sigma, Deisenhofen, Germany) was added to the medium and absorbance was determined at 570 and 630 nm (reference) on a microplate reader (ELx800, Biotek Instruments, Heidelberg, Germany).

Evaluation of drug interactions

To calculate combined drug effects, the combination index (CI) isobologram method of Chou and Talalay was used [4, 5, 6, 8, 9]. This method involves plotting dose-effect curves for each agent and combinations thereof in multiply-diluted concentrations by using the median-effect equation and plot [5] and the CI equation and plot [8, 9]. The method takes into account not only the potency of each drug and their combinations (D_m values), but also the shape of their dose-effect curves (m values), and determines how much the experimental effect differs from the effect expected with additivity. CI values 1, <1, and >1 indicate an additive effect, synergism or antagonism, respectively. The CI values can be determined at different effect levels and different dose levels, and the isobolograms can be automatically generated by using the computer software CalcuSyn [7]. The dose-reduction index (DRI) is determined by comparing the ratio of the doses required to reach a given degree of growth inhibition for a single drug and for each drug in the combination.

Results

TMZ, TX and DX as single agents or in combinations inhibited significantly the growth of the human malignant glioma cell lines U87MG, T98G, LNZ308, and wt1119 in a concentration-dependent manner (Fig. 1, Table 1). The combination effects of TMZ, TX and DX in human glioma cell lines, as represented by the DRI, the CI and the dose-effect levels of cell growth inhibition (ED_{50}–ED_{95}), are summarized in Table 1. In U87MG cells, IC_{50} values for DX, TX and TMZ were 252 μM, 203 μM and 195 μM, respectively. In T98G cells, the IC_{50} values for DX, TX and TMZ were 35 μM, 198 μM and 1446 μM, and in LNZ308 cells, 98 μM, 147 μM and 988 μM, respectively. Finally, in wt1119 p53-positive cells, the IC_{50} values for DX, TX and TMZ were 132 μM, 173 μM and 2498 μM, and in the corresponding wt1119 p53-null cells, 122 μM, 133 μM and 2936 μM.

Compared with TMZ, TX and DX showed greater cytotoxicity in all cell lines at equimolar concentrations. DX was the most potent single drug. The cytotoxic effect of TX at low drug concentrations was weaker than that of DX. At higher concentrations, the cytotoxicity of TX increased and resulted in almost complete elimination of tumor cells (Fig. 1). The combination TMZ/DX in U87MG, T98G, LNZ308, wt1119 p53-positive and wt1119 p53-null cells showed IC_{50} values of $85+21$ μM, $267+67$ μM, $233+58$ μM, $350+87$ μM and $326+81$ μM, respectively. The combination TMZ/TX in U87MG, T98G, LNZ308, wt1119 p53-positive and wt1119 p53-null cells showed IC_{50} values of $71+18$ μM, $327+82$ μM, $283+71$ μM, $450+112$ μM and $427+107$ μM, respectively (Table 1).

In T98G cells, combinations were synergistic at low dose levels (ED_{50}) for the combination TMZ/TX, or at high levels (ED_{90} and ED_{95}) for TMZ/DX. In LNZ308 and wt1119 p53-negative and p53-positive cells, syner-

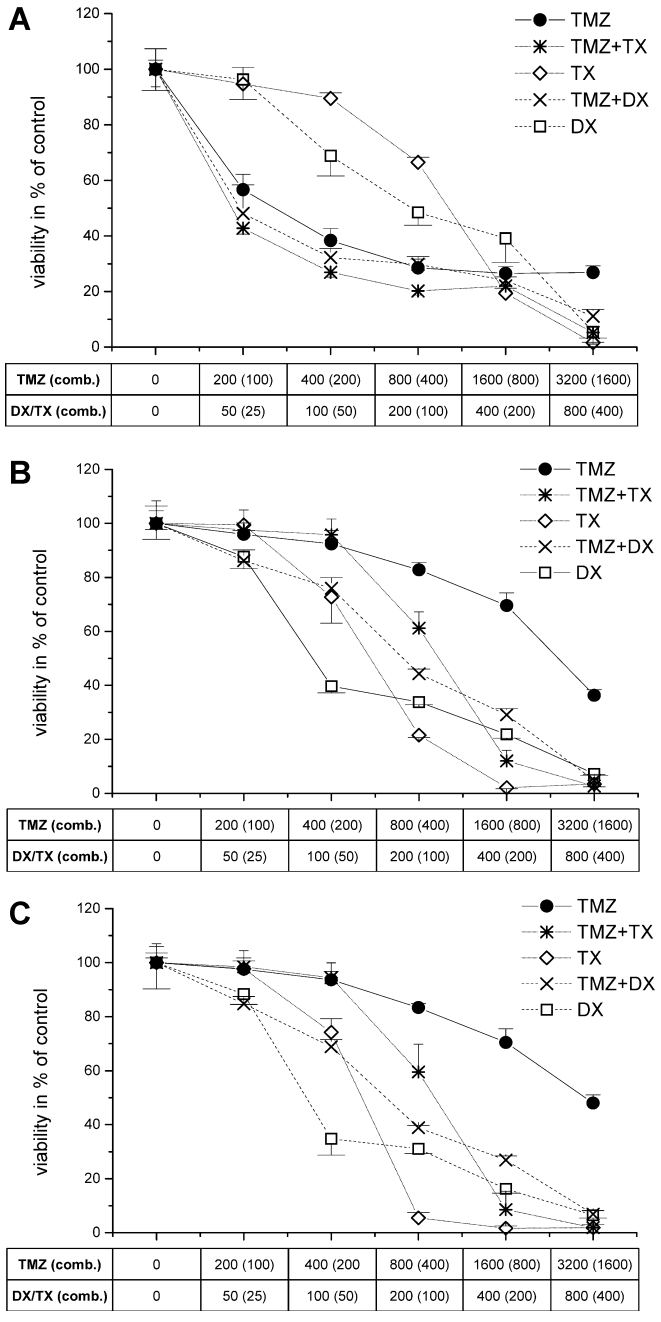

Fig. 1A–C Representative dose-response curves for TMZ, TX, and DX, and their combinations in U87MG (**A**), wt1119 wtp53-expressing (**B**), and wt1119 p53-deficient (**C**) human malignant glioma cells. There was no significant difference in the dose-response effect of drugs on wt1119 cells in the presence or absence of wild-type p53 protein. Concentrations are given in micromoles. *Numbers in parentheses* indicate drug doses when used in combinations

gistic effects were recorded at all dose levels for both combinations (Fig. 2, Table 1). Synergistic effects were also demonstrated in U87MG cells, except at high dose levels (ED_{95} for TMZ/TX, and ED_{90} and ED_{95} for TMZ/DX; Table 1).

The DRI showed a considerable dose reduction for all drugs used as a result of their synergism (Table 1). When using synergistic drug combinations at the corresponding dose levels, the DRI indicated that the concentration of TMZ necessary to inhibit the growth of 50% of glioma cells (ED_{50}) could be decreased 2.28-fold (U87MG, TMZ/DX) to 5.42-fold (T98G, TMZ/DX), and the ED_{95} could be reduced 1.84-fold (T98G, TMZ/TX) to 98.8-fold (LNZ308, TMZ/TX; Table 1). The dose reduction level was different and specific to each cell line.

Discussion

In this study, the toxicities of the alkylating drug TMZ and the RR inhibitors DX and TX were investigated in human malignant glioma cells. TMZ combined with DX or TX resulted in synergistically enhanced cytotoxicity at different dose levels in all glioma cell lines. Calculation of the DRI at the IC_{50} demonstrated possible reductions in TMZ concentrations for the drug combinations ranging from 2.28-fold (TMZ/DX in U87MG) to 5.42-fold (TMZ/DX in T98G). The toxicity of the drugs used and the presence of drug synergy did not correlate with p53 status, as demonstrated in a wt*p53* transgene system (Tet-off) in wt1119 glioma cells.

Ribonucleotide reductase inhibitors

RR inhibition has been shown previously to result in depletion of DNA precursors and consequently in a block of DNA synthesis [20]. A number of compounds inhibiting RR are already in clinical use, such as hydroxyurea, gemcitabine, and fludarabine. A new group of RR inhibitors are the polyhydroxy-substituted benzoic acid derivatives. Among these, TX and DX are the most powerful enzyme inhibitors demonstrating excellent anticancer activity in animal tumor models [13]. TX and DX deplete dNTP pools and inhibit the growth of various tumor cell lines more effectively than hydroxyurea. As shown recently, depletion or imbalance of dNTPs (dCTP and dGTP) can induce apoptosis [13]. It has also recently been demonstrated that TX synergistically enhances the metabolism of Ara-C in leukemia cells. Preincubation with TX significantly increases the incorporation of Ara-C metabolites into DNA and hence amplifies its toxicity [14].

In our experimental series, both DX and TX were very effective in inhibiting the growth of malignant glioma cells, although the actual tumor cell toxicity of the two drugs varied considerably in different glioma cell lines with their different genetic alterations and presumably different resistance mechanisms. A possible mechanism of resistance to RR inhibitors has been described for gemcitabine, a deoxycytidine competitor used in the treatment of pancreatic and non-small-cell lung cancer. Inhibition of RR by gemcitabine diphosphate results in reduced production of dNTPs. Overexpression of RR leads to an increased dNTP pool size,

Table 1 Dose-effect relationships of single drugs and combinations in human malignant glioma cell lines. Dose-effect relationships were calculated by the median-effect equation [4, 5]. D_m median-effect dose (concentration in micromoles that inhibits cell growth by 50%), *m* shape of the dose-effect curve (where m = 1, m > 1, and m < 1 indicate hyperbolic, sigmoidal, and negative sigmoidal curves, respectively), *r* linear correlation coefficient of the median-effect plot (indicates conformity of data). CI was calculated by the CI equation of Chou and Talalay [7, 9]. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. D_m and *m* values for single drugs and their combinations were used in the equations $D_x = D_m [f_a/(1-f_a)]^{1/m}$ and $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$. f_a fraction affected by D (e.g. 0.9 if cell growth is inhibited by 90%), $(D)_1$ and $(D)_2$ combined doses of drug 1 and drug 2 for *x*% inhibition, $(D_x)_1$ and $(D_x)_2$ doses of the single drugs 1 and 2 for *x*% inhibition. *DRI* dose reduction index measured by comparing the doses required to reach a given degree of inhibition when using the drug as single agent and in combination. Drugs were combined at a molar ratio of 4:1(TMZ/DX or TX)

Cell line	Single drugs and combinations	Parameters			CI value at				DRI value at			
		D_m	m	r	ED_{50}	ED_{75}	ED_{90}	ED_{95}	ED_{50}	ED_{75}	ED_{90}	ED_{95}
T98G	TMZ	1446.3	1.62	0.98					4.43	3.18	2.29	1.84
	TX	198.1	2.17	0.96					2.24	1.47	0.89	0.63
	TMZ + TX(4:1)	327.3 + 81.8	1.09	0.99	0.64	0.99	1.56	2.12				
	TMZ	1446.3	1.62	0.98					5.42	4.71	4.10	3.72
	DX	34.7	0.79	0.96					0.52	0.93	1.65	2.44
	TMZ + DX(4:1)	266.9 + 66.7	1.34	0.99	2.11	1.29	0.85	0.68				
U87MG	TMZ	194.6	0.45	0.89					2.75	8.04	23.52	48.83
	TX	202.7	2.53	0.98					11.44	4.38	1.68	0.87
	TMZ + TX(4:1)	70.9 + 17.7	0.79	0.91	0.45	0.35	0.64	1.17				
	TMZ	194.6	0.45	0.89					2.28	4.78	10.04	16.64
	DX	251.9	2.62	0.92					11.79	3.19	0.87	0.36
	TMZ + DX(4:1)	85.5 + 21.4	0.64	0.96	0.52	0.52	1.25	2.87				
LNZ308	TMZ	987.7	0.57	0.93					3.49	12.15	42.28	98.80
	TX	147.2	2.48	0.97					2.08	1.66	1.32	1.13
	TMZ + TX(4:1)	283.1 + 70.8	1.64	0.93	0.77	0.69	0.78	0.89				
	TMZ	987.7	0.57	0.93					4.23	10.88	27.90	52.90
	DX	97.8	1.22	0.98					1.68	1.56	1.46	1.38
	TMZ + DX(4:1)	232.7 + 58.2	1.13	0.99	0.83	0.73	0.72	0.74				
wt1119 (no wtp53)	TMZ	2935.6	1.35	1.00					6.87	10.84	17.11	23.35
	TX	133.1	2.98	0.94					1.25	1.26	1.28	1.29
	TMZ + TX(4:1)	427.4 + 106.8	3.09	0.99	0.95	0.88	0.84	0.82				
	TMZ	2935.6	1.35	1.00					9.00	9.79	10.63	11.24
	DX	121.9	1.50	0.94					1.50	1.50	1.50	1.51
	TMZ + DX(4:1)	325.6 + 81.4	1.50	0.99	0.78	0.77	0.76	0.76				
wt1119 (wtp53)	TMZ	2498.1	1.32	0.99					5.55	8.69	13.59	18.43
	TX	172.9	3.16	0.94					1.54	1.48	1.42	1.39
	TMZ + TX(4:1)	449.8 + 112.4	2.83	0.99	0.83	0.79	0.78	0.78				
	TMZ	2498.1	1.32	0.99					11.14	8.57	10.28	11.63
	DX	132.1	1.42	0.95					1.51	1.70	1.91	2.09
	TMZ + DX(4:1)	349.8 + 87.4	1.68	0.98	0.80	.071	0.62	0.57				

which in turn results in downregulation of deoxycytidine kinase (DCK) via negative feedback. Excess dCTP may activate dCMP deaminase via positive feedback resulting in increased gemcitabine metabolism [15]. There is, however, no published evidence suggesting that this mechanism is actually active in glioma cells.

Temozolomide

TMZ penetrates the CNS and does not require hepatic metabolism for activation, since in vivo it undergoes spontaneous pH-dependent conversion to the active alkylating agent MTIC [11]. MTIC in turn degrades to a methyldiazonium cation and methylates DNA. The cytotoxicity of TMZ appears to be mediated mainly through O(6)-methylguanine adducts in genomic DNA and may be reversed by the action of the enzyme O(6)-methylguanine-DNA methyltransferase (MGMT) [1, 11]. Methylation of O(6)-guanine seems to be necessary for triggering apoptosis through activation of the mismatch-repair (MMR) system in TMZ-treated cells, since the process is prevented by high MGMT levels [25]. MGMT has been implicated in mediating resistance of human brain tumors to alkylating compounds, such as TMZ, whose toxicity in human tumor cell lines negatively correlates with the level of expression of MGMT [16, 18]. Variable and inducible levels of MGMT may have been at least in part responsible in our experiments for the variability of growth inhibition caused by TMZ in different glioma cell lines.

Interactions between TMZ and RR inhibitors

The most prominent effect of TMZ in glioma is G_2/M arrest. This arrest is initiated in a p53-independent manner, but p53 seems to influence its duration [16]. G_2/M arrest in response to TMZ may protect cells from TMZ-induced cytotoxicity and may therefore represent

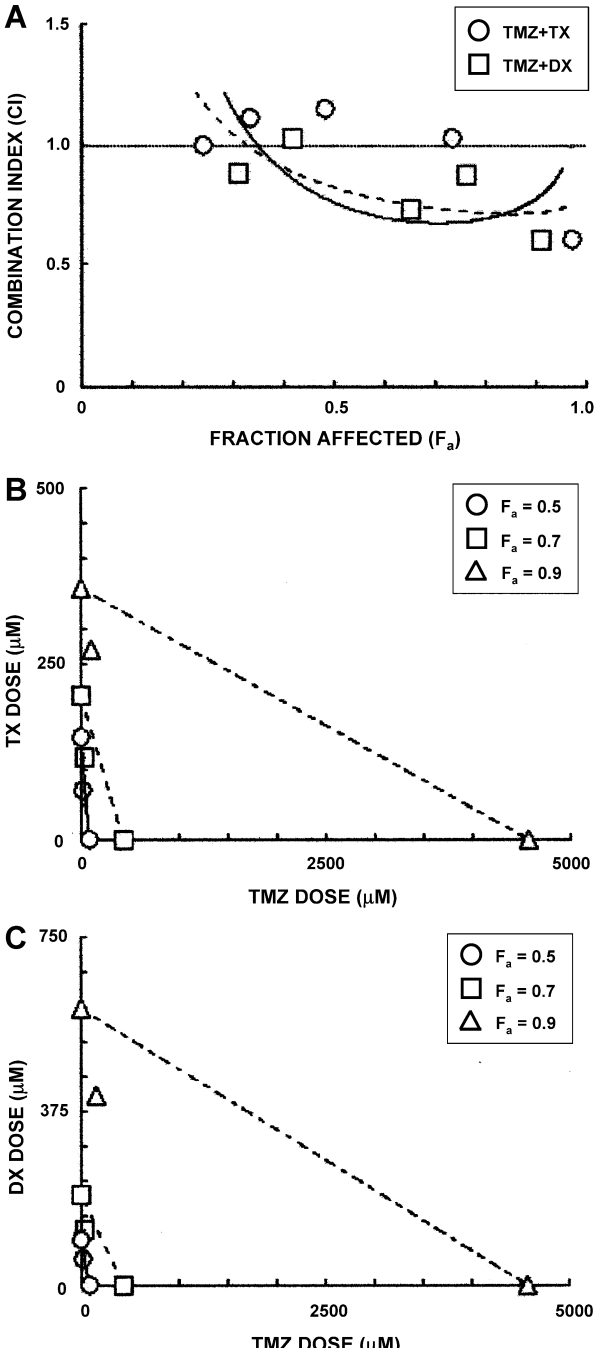

Fig. 2A–C Analysis of the combinations of TMZ with DX/TX in LNZ308 cells. **A** CI plot for the combinations of drugs (TMZ + DX/TX 4:1); **B** isobologram for the combination of TMZ with TX at different effect levels (F_a); **C** isobologram for the combination of TMZ with DX at different effect levels (F_a). Note that the combination data points in (**B**, **C**) fall on the lower-left of the hypotenuse of each effect level and therefore indicate synergism

a mechanism of drug resistance. Since the G_2/M arrest provides an opportunity for tumor cells to reduce TMZ-induced cytotoxicity, a premature (G_1/S) arrest by relatively low doses of DX/TX may expose cells to TMZ in a more vulnerable phase of the cell cycle.

A further and more important mechanism for synergistic cytotoxicity of RR inhibitors and TMZ may be the inhibition of repair of TMZ-induced DNA damage by depletion of precursors (dNTP). As different glioma cell lines differ in their repair potential, which in addition may change over time, drug effects may also vary. On the other hand, inhibition of DNA synthesis by DX or TX may increase the time available for DNA repair in response to TMZ-mediated damage, which could in turn explain drug antagonisms at certain, usually high, dose levels (e.g. ED_{90} and ED_{95}).

In the present study, the combination of TMZ with RR inhibitors enhanced the action of TMZ not only in wtp53-expressing but also in wtp53-deficient cells. Because approximately two-thirds of gliomas have defects in the p53 pathway, the ability to affect cells regardless of their p53 status also increases the range of tumors for which this approach might be effective. Whereas issues relating to the molecular events that link TMZ action to DX/TX effects remain to be examined, this study suggests that combination of the alkylating agent TMZ with RR inhibitors, such as DX or TX, might represent an alternative option for enhancement of its toxic effect on brain tumor cells. In an in vivo setting, another possible advantage of combining TMZ with RR inhibitors could be the reduction of toxic side effects of TMZ, such as myelosuppression, by decreasing the effective dose of TMZ as indicated by the DRI (Table 1).

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