The Co-Treatment of Temozolomide and Doxycycline Exhibits Antagonism Against Human Glioblastoma U87 Cells: A Short Communication

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Abstract
One of the treatment modalities in cancers, including glioblastoma (GB), is drug combinations. Nonetheless, the combined effect can be equal to, less than, or greater than each drug alone, and an effect greater than either agent alone does not necessarily imply synergism. This study evaluated the cytotoxic interaction of temozolomide (TMZ) and doxycycline (DOXY) co-treatment in the human GB U87 cell line. U87 cells were treated with TMZ, DOXY, and TMZ+DOXY (0.5 to 225.0 µg/ml) for 48 h. Cytotoxic effects were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data were analysed by two-way ANOVA (p < 0.05, two-tailed), followed by Tukey’s post hoc test. Drug interaction was based on combination index (CI) values, where < 0.9, 0.9-1.1, and > 1.1 represent synergistic, additive, and antagonistic effects, respectively. TMZ+DOXY cytotoxicity ranged from potentiated (0.5 to 105.0 µg/ml) to diminished (135.0 to 225.0 µg/ml), with statistically significant differences at concentrations of 7.5, 15.0, and 75.0 µg/ml when compared to individual treatments. However, CI values were all greater than 1.1. TMZ and DOXY co-treatments in U87 cells exhibit antagonistic cytotoxicity, which may not be viable clinically.

Keywords
Temozolomide, Doxycycline, Co-treatment, Glioblastoma cells, Antagonistic interaction
Introduction
Glioblastoma (GB) is a type of high-grade glioma, also known as WHO grade IV astrocytoma. It is the most frequently diagnosed malignant primary brain tumor in adults and the most devastating type with a dismal prognosis and survival [1]. The first-line chemotherapeutic agent for GB is temozolomide (TMZ); however, resistance to it is expected. Indeed, this glial-derived tumor, which comprises neoplastic and stromal elements, exhibits remarkable phenotypic heterogeneity and plasticity, leading to variable treatment outcomes, as well as presenting a key limitation to sustaining treatment response. To address these challenges, enormous efforts have been made, including an attempt to achieve synergistic anti-GB effects in order to enhance potency and efficacy by combining TMZ with various types of agents.

DOXY, a second-generation tetracycline-class antibiotic, is one of the appealing candidates not only because of its favourable pharmacokinetic profile, but also affordability and potentiality. It is known to inhibit mitochondrial biogenesis and oxidative phosphorylation, which renders therapy-resistant, particularly in cancer stem cells. This subpopulation of tumor cells is therefore vulnerable to DOXY-induced cytotoxicity as they rely on mitochondria for survival, propagation, and stemness maintenance [3, 4]. Moreover, it has been shown effective in inhibiting cell proliferation and inducing apoptosis in a variety of cancer models, as well as hampering other features (processes) that support tumor growth, survival, and progressions, such as angiogenesis, autophagy, epithelial-mesenchymal transition, and inflammation [5-8].

In vitro and in vivo drug combination interactions follow the same principle, with in vitro investigations usually performed first, followed by in vivo and clinical development [2]. Although the combined effects of TMZ and DOXY have been reported, their interaction is still unclear. The objective of this study was to determine the cytotoxic interaction of TMZ and doxycycline (DOXY) against human GB U87 cells.

Materials and Methods
Cell culture
U87 cells (American Type Culture Collection, Rockville, USA) were grown in Dulbecco’s modified Eagle’s medium containing glucose (4.5 g/L), sodium pyruvate, and L-glutamine (Nacalai Tesque, Japan), supplemented with 1% nonessential amino acids (Nacalai Tesque, Japan) and 5% foetal bovine serum (Tico Europe, Netherlands) under standardised conditions (5% CO2 at 37°C/95% humidity).

Drug combination analysis
U87 cells (5000/100 µl per well) were seeded in 96-well flat-bottomed plates (SPL Life Sciences, Korea) and incubated for 24 h before being treated with TMZ, DOXY, and TMZ+DOXY (0.5 to 225.0 µg/ml). Following 48 h, cell viability was assayed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Invitrogen, Thermo Fisher Scientific, USA) as directed by the manufacturer, and the absorbance was measured at 540 nm using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, USA). The inhibition percentage of cell viability was calculated as follows: 100 - [(mean absorbance of treated cells – mean absorbance of blank)/mean absorbance of untreated cells – mean absorbance of blank) × 100]. The interaction between two drugs was determined based on the CI values estimated as follows: CI = Inhibition (%) (TMZ+DOXY/TMZ) + (TMZ+DOXY/DOXY) [2].

Statistical analysis
Data were normally distributed as determined by the Shapiro-Wilk normality test (p > 0.05). A statistically significant two-way ANOVA (p < 0.05, two-tailed) was followed by Tukey’s post hoc comparisons using GraphPad Prism version 6.01 (GraphPad Software, CA, USA).
Figure 1. U87 cells were treated with TMZ, DOXY, and TMZ+DOXY (0.5 to 225.0 µg/ml) for 48 h, followed by MTT cytotoxicity assay; (a) concentration-effect plot and (b) CI plot of manually calculated drug interaction using the equation: CI = inhibition (%) (TMZ+DOXY/TMZ) + (TMZ+DOXY/DOXY) [2]. The difference was statistically significant when compared to the TMZ (*) and DOXY (†) (*p < 0.05, **p < 0.01). The CI values <0.9, 0.9-1.1, and >1.1 represent synergistic, additive (dashed lines in the graph), and antagonistic effects, respectively. The Shapiro-Wilk normality test and two-way ANOVA, followed by Tukey's post hoc comparisons were performed using GraphPad Prism version 6.01 (GraphPad Software, CA, USA). Data are represented by the arithmetic mean (standard deviation) of three independent repeats.

Results and Discussion
DOXY has been shown to be nearly as cytotoxic as TMZ against U87 cells, with half-maximal inhibitory concentrations of 147.0 and 151.0 µg/ml, respectively [8]. Herein, the cytotoxicity of TMZ+DOXY in U87 cells increased at concentrations ranging from 0.5 to 105.0 µg/ml but then decreased at concentrations ranging from 135.0 to 225.0 µg/ml, when compared to individual treatments (Figure 1 a). There was
Evidence of statistically significant differences at concentrations of 7.5, 15.0, and 75.0 µg/ml when compared to individual treatments. CI analysis, on the other hand, delineated that TMZ+DOXY treatment had an antagonistic interaction at all concentrations tested (Figure 1b).

In a different study, co-treatment with TMZ and DOXY resulted in synergistic cytotoxicity in U373-U GB cells, but whether antagonistic in U87 and U251 is unspecified [9]. Tan and colleagues revealed that TMZ and DOXY had essentially equivalent antiproliferative effects in vitro (U87 and A172 GB cells) and in vivo (A172 xenograft SCID mice), whilst TMZ+DOXY outperformed both [10]. Another group of scientists found that TMZ+DOXY-treated primary GB cells achieved cytotoxic and antiproliferative effects close to DOXY alone; however, were more effective than TMZ [11].

It is noteworthy that DOXY+rapamycin cytotoxicity was synergistic in U251 and U373-U GB cells, but merely an additive effect in U87 [9]. DOXY demonstrated antagonism in MDA-MB-231 breast cancer cells when combined with chemotherapeutic drugs, such as doxorubicin and paclitaxel, whereas synergy and additivity with cisplatin at high and low concentration levels, respectively [12]. In PANC1 pancreatic cancer cells, DOXY acted synergistically with cisplatin, oxaliplatin, sorafenib, gemcitabine, and 5-fluorouracil [13].

Conclusion
In a nutshell, this report provides evidence of in vitro antagonistic cytotoxicity of TMZ and DOXY co-treatment in U87 cells. Alternatively, it may be sensible to explore whether DOXY can increase the sensitivity of GB cells to TMZ when given sequentially.

Abbreviations
GB: glioblastoma; TMZ: temozolomide; DOXY: doxycycline; co-treatment: combination treatment; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; CI: combination index; ANOVA: analysis of variance.

Competing interests
All authors stated that they have no competing interests regarding the publication of this manuscript.

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