



Cytotoxicity screening of cotreatment with temozolomide and azithromycin on human glioblastoma cells U87

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ABSTRACT

Drug combinations can improve the efficacy of anticancer therapy and reduce the side effects. Our preliminary data suggest that clinically used antibiotics such as azithromycin (AZI) reduce proliferative capacity and induce apoptosis in human glioblastoma (GBM) cells U87. Hence, the present study explored the cytotoxicity of cotreatment with temozolomide (TMZ) and AZI on U87 cells. The cells were treated with each drug individually and in combination in a series of concentrations (0.5–225.0 µg/ml) for 48 h synchronously. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and data were analyzed with the Code for the Identification of Synergism Numerically Efficient software. The cytotoxicity was potentiated when the cells were cotreated with TMZ and AZI compared to TMZ alone, but was markedly lower than AZI alone. Moreover, it was shown that TMZ + AZI exerted a competitive inhibition of cell growth and TMZ partially repressed the cytotoxicity of AZI. These findings delineate that the TMZ and AZI cotreatment behaves antagonistically in human GBM cells U87. A further chapter on the pretreatment of cells with AZI prior to TMZ or a so-called sensitization strategy may provide valuable insight into the fight against GBM.

INTRODUCTION

Temozolomide (TMZ) chemotherapy remains the keystone treatment modality for patients with glioblastoma (GBM). Although the administration of concomitant and adjuvant TMZ along with radiotherapy has achieved longer-term survival, its efficacy is limited by a wide range of aspects (Chen *et al.*, 2012; Stupp *et al.*, 2005). As such, numerous efforts are being made to improve the efficacy of TMZ and one of the current horizons is the combination of drugs. This approach to drug design has been supported by encouraging clinical benefits, such as sequential bortezomib and TMZ (Rahman *et al.*, 2020).

The nonantibiotic properties of azithromycin (AZI) have been shown to be active and effective against cancer cells and cancer stem-like cells (CSCs) of multiple human cancer types (Lamb *et al.*, 2015; Qiao *et al.*, 2018; Zhou *et al.*, 2012). The combination of AZI and tumor necrosis factor-related apoptosis-inducing ligand obtained synergistic cytotoxicity in colon cancer cells *in vitro* and *in vivo* (Qiao *et al.*, 2018). AZI also has selective cytotoxicity to cancer cells and synergistic cytotoxicity with the chemotherapeutic agent vincristine in cervical and gastric cancer cells *in vitro* (Zhou *et al.*, 2012). Besides, the 1-year survival rate of advanced non-small-cell lung cancer patients demonstrated an increase with the addition of AZI to the chemotherapy regimen (Chu *et al.*, 2014).

Screening to understand the properties of drugs in combination is a fundamental element in the development of anticancer combination therapies. In this study, the cytotoxicity of cotreatment with TMZ and AZI on human GBM cells U87 was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and drug interactions

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were analyzed by the Code for the Identification of Synergism Numerically Efficient (CISNE) software.

MATERIALS AND METHODS

Drugs

TMZ (CAS Number: 85622-93-1, Sigma-Aldrich, St. Louis, MO) and AZI (CAS Number: 83905-01-5, St. Louis, MO) were dissolved in dimethyl sulfoxide (CAS Number: 67-68-5, St. Louis, MO).

Cell line

The human GBM cells U87 MG (HTB-14 Lot number: 63710285) were purchased from the American Type Culture Collection (Rockville, MD). Cells 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% fetal bovine serum (Tico Europe, Netherlands) under standardized conditions (5% CO₂ at 37°C/95% humidity).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Five thousand cells with a final volume of 100 µl were seeded in 96-well flat bottomed plates (SPL Life Sciences, Korea) and were incubated for 24 hours. Then, the cells were treated with TMZ, AZI, and a combination thereof in a series of concentrations (0.5–225.0 µg/ml). After 48 hours, MTT (Invitrogen, Thermo Fisher Scientific, Waltham, MA) assay was carried out as instructed by the manufacturer and the absorbance was taken at a wavelength of 540 nm using the Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA). The percentage of cell viability or a fraction of unaffected (Fu) cells was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{(\text{absorbance of treated cells} - \text{absorbance of blank})}{(\text{absorbance of untreated cells} - \text{absorbance of blank})} \times 100$$

Drug combination analysis

To determine the cytotoxic interactions between TMZ and AZI, CISNE software version 1.0 (Universidad de Oviedo, Spain) (García-Fuente *et al.*, 2018) was used to compute the combination index (CI) and the isobologram. Synergistic, additive, and antagonistic interactions are indicated by CI values of < 0.8, 0.8–1.2, and > 1.2, respectively. The isobologram represents the concentration of TMZ and AZI on the x- and y-axis, respectively. A straight red line joins the half-maximal inhibitory concentration (IC₅₀) of each drug and dashed red lines demonstrate the upper and lower limits of a 95% confidence interval. The IC₅₀ in the combination is plotted as a blue dot, with the confidence interval of both drug concentrations represented by a dashed blue line in an elliptical shape.

RESULTS AND DISCUSSION

Drug interactions are an important aspect of anticancer drug design. Scientists have gone the extra mile to achieve synergistic efficacy of various drug combinations because synergistic-acting combinations not only increase the effect but also decrease the dose. Besides, interactions between drugs in combination can also be classified as either antagonistic or additive. Antagonistic combinations generate lower effects than the additive because one drug hinders the efficacy of another. Alternatively, the additive occurs when the drugs do not interact with each other, and therefore, the effect is neither more nor less than individually (Chou, 2006; Zimmermann *et al.*, 2007).

In the present study, cotreatment of the human GBM cells U87 with TMZ and AZI ameliorates the cytotoxicity in a dose-dependent manner (Fig. 1). However, the inhibitory effect improved slightly compared to TMZ alone, while it was markedly lower than AZI alone. The concentration of TMZ + AZI that caused 50% of the cells to be affected (IC₅₀) was 121.77 ± 25.19 µg/ml, compared to TMZ and AZI individually at 201.22 ± 48.78 µg/ml and 61.80 ± 27.88 µg/ml, respectively (Table 1). Specifically, drug interaction analysis evidenced an antagonistic interaction between TMZ and AZI (Fig. 2), which accounted for the observed competitive inhibition of cell viability (Fig. 1). This discovery, therefore, postulates that the alkylating agent (TMZ) and the

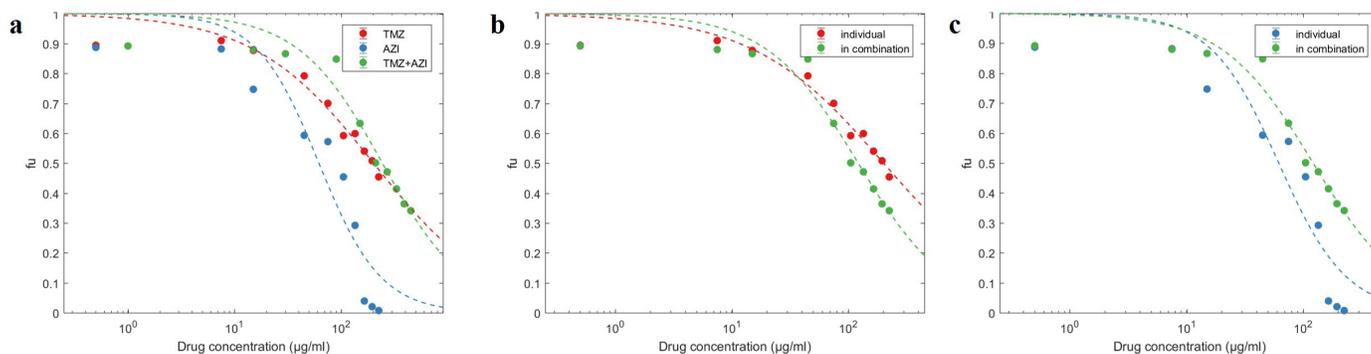


Figure 1. Dose-effect fitting obtained for TMZ + AZI cotreatment compared to each drug alone. (a) TMZ + AZI dose-effect curve comparison, (b) TMZ dose-effect change in combination, and (c) AZI dose-effect change in combination. Data represent the mean ± standard deviation (SD) of three independent experiments carried out in triplicate.

Table 1. Antagonistic cytotoxicity of cotreatment with TMZ and AZI on U87 cells for 48 hours.

TMZ ($\mu\text{g/ml}$)	AZI ($\mu\text{g/ml}$)	Fu	CISNE-generated parameters		CISNE-simulation	
			m	D	Fa	CI
0.50		0.89	0.77 ± 0.22	201.22 ± 48.78	0.05	2.87
7.50		0.91			0.10	2.59
15.00		0.88			0.15	2.49
45.00		0.79			0.20	2.45
75.00		0.70			0.25	2.45
105.00		0.59			0.30	2.45
135.00		0.60			0.35	2.47
165.00		0.54			0.40	2.50
195.00		0.51			0.45	2.53
225.00		0.45			0.50	2.58
	0.50	0.89	1.49 ± 0.94	61.80 ± 27.88	0.55	2.63
	7.50	0.88			0.60	2.69
	15.00	0.75			0.65	2.76
	45.00	0.59			0.70	2.84
	75.00	0.57			0.75	2.95
	105.00	0.45			0.80	3.09
	135.00	0.29			0.85	3.28
	165.00	0.04			0.90	3.57
	195.00	0.02			0.95	4.15
	225.00	0.01				
0.25	0.25	0.89	1.10 ± 0.39	121.77 ± 25.19		
3.75	3.75	0.88				
7.50	7.50	0.87				
22.50	22.50	0.85				
37.50	37.50	0.63				
52.50	52.50	0.50				
67.50	67.50	0.47				
82.50	82.50	0.41				
97.50	97.50	0.36				
112.50	112.50	0.34				

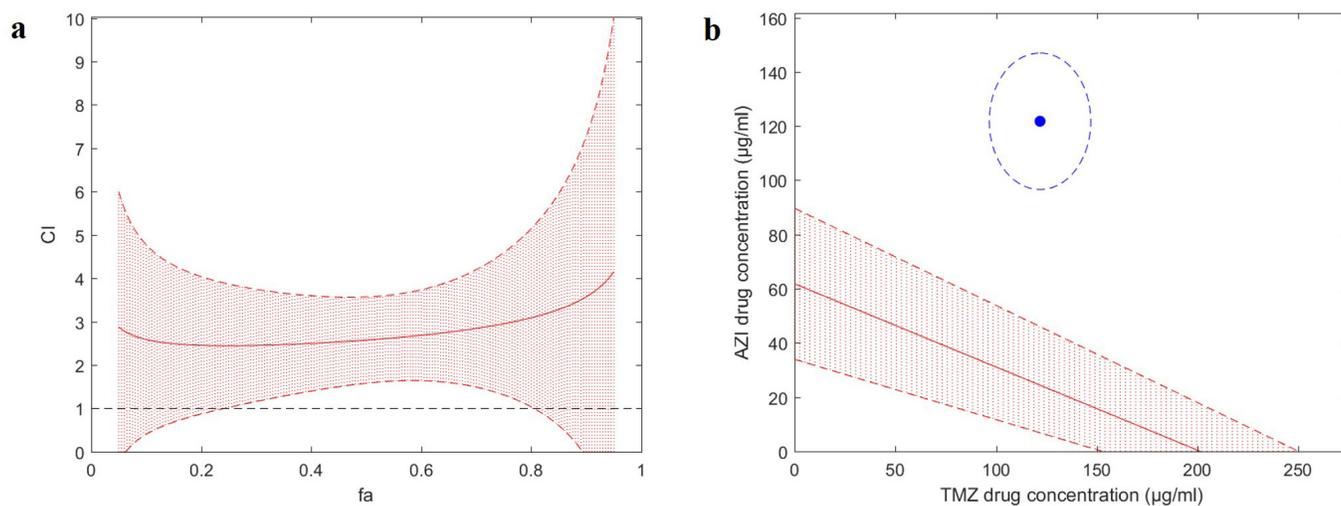


Figure 2. CISNE-generated (a) Fa-CI plot and (b) isobologram for TMZ + AZI cotreatment. The red line with the 95% confidence interval (red-shaded region) in the Fa-CI plot above the black dashed line indicates an antagonistic interaction between TMZ and AZI. The isobologram, wherein the red line represents an additive effect, is synergistic if the blue dot within the space is limited by the red line and the axes, antagonistic if the blue dot is outside the triangle, and additive if the confidence intervals overlap.

macrolide antibiotic (AZI) may compete for the same molecular (protein) target and cause cell death by the same mechanism in U87 cells. Indeed, studies have shown that TMZ and AZI can exert their cytotoxic activity, partly by mitochondrial-mediated caspase-dependent apoptosis (Lamb *et al.*, 2015; Liu *et al.*, 2018; Zhou *et al.*, 2012). In a different study, the combination of TMZ and curcumin (a bioactive compound from turmeric) showed an additive effect on GBM cells *in vitro* and tumor growth *in vivo* due to redundant cytotoxicity mechanisms (Zanotto-Filho *et al.*, 2015).

According to a competitive binding model, two drugs will be antagonistic if they target similar sites for the same protein (Krogstad *et al.*, 1986). Drug combinations targeting the same protein have also been delineated as antagonistic companions (Cokol *et al.*, 2011). Furthermore, a genetic epistatic interaction study suggests that two drugs will be synergistic if they inhibit different molecules or pathways of interconnected networks that serve the same purpose (Yeh *et al.*, 2009). Certainly, synergistic-acting combinations rarely have similar or directly linked targets characterized by a network-based computational method (Zou *et al.*, 2012).

It is known that the use of drugs in combination can effectively control cancers by simultaneously thwarting a number of targets and making them less susceptible to resistance (Qiao *et al.*, 2018; Yeh *et al.*, 2009; Zimmermann *et al.*, 2007). While synergistic efficacy is pressingly desired in this realm, drug combinations with antagonistic interaction may not be disregarded. In one study, the antagonistic combination is capable of offering a long-term constraint on the rise of therapeutic resistance compared to the synergistic combination (Saputra *et al.*, 2018). Similarly, in the perspective of antimicrobials, antagonistic combinations can select antibiotic-resistant bacteria and minimize the evolution of resistance (Yeh *et al.*, 2009). Herein, TMZ partially suppressed the cytotoxicity of AZI in U87 cells (Table 1) (see also Supplementary File 1). Taking into account the fact that AZI was found to be active and effective against both non-CSCs and CSCs, while TMZ was not (Chen *et al.*, 2012; Lamb *et al.*, 2015; Qiao *et al.*, 2018; Zhou *et al.*, 2012), it may be helpful to target therapy-resistant GBM by combining both drugs.

Additionally, sensitization is another strategy that can enhance the efficacy of anticancer therapy and reduce the side effects (Ciechomska *et al.*, 2018; Matsuno *et al.*, 2018). An *in vitro* investigation uncovered that pretreatment and posttreatment with a small-molecule inhibitor for the histone methyltransferase G9a (a biomarker associated with cancer progression and metastasis) sensitized GBM cells to TMZ and increased the efficacy of TMZ (Ciechomska *et al.*, 2018). From now on, alternative ways, such as pretreatment, to evaluate whether AZI can sensitize cells to TMZ could be an approach worth exploring.

CONCLUSION

Cotreatment with TMZ and AZI exhibits antagonistic cytotoxicity in human GBM cells U87 between low and middle concentration ranges. This finding warrants further investigation using a number of different GBM cell lines, including glioma stem-like cells.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

Not applicable.

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Supplementary File 1.

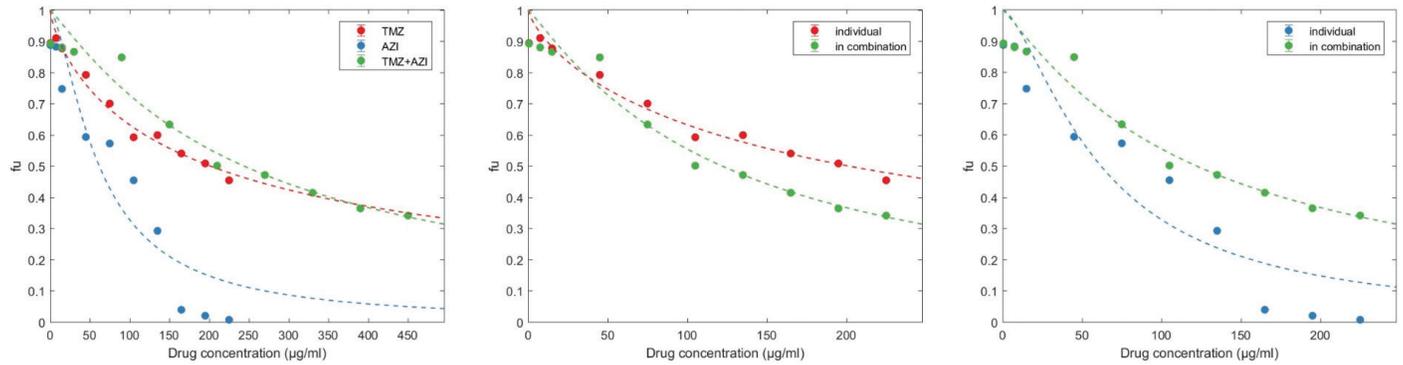


Figure 1. CISNE-generated dose-effect curve for TMZ + AZI cotreatment compared to each drug individually.



Figure 2. Effect of TMZ on U87 cells. Microphotographs were taken after 48h of incubation with different concentrations of TMZ (scale bars = 100 µm).

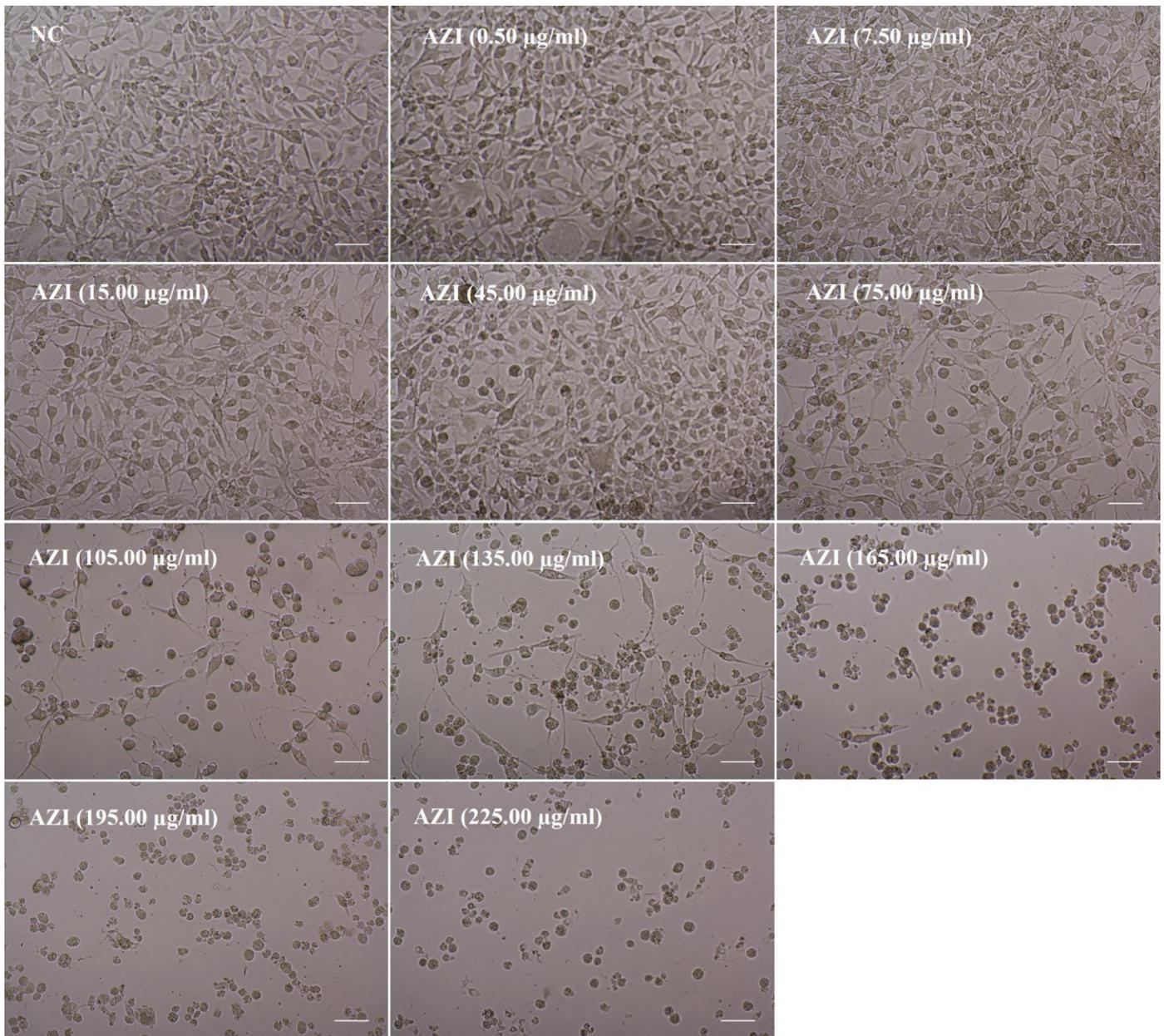


Figure 3. Effect of AZI on U87 cells. Microphotographs were taken after 48h of incubation with different concentrations of AZI (scale bars = 100 µm).

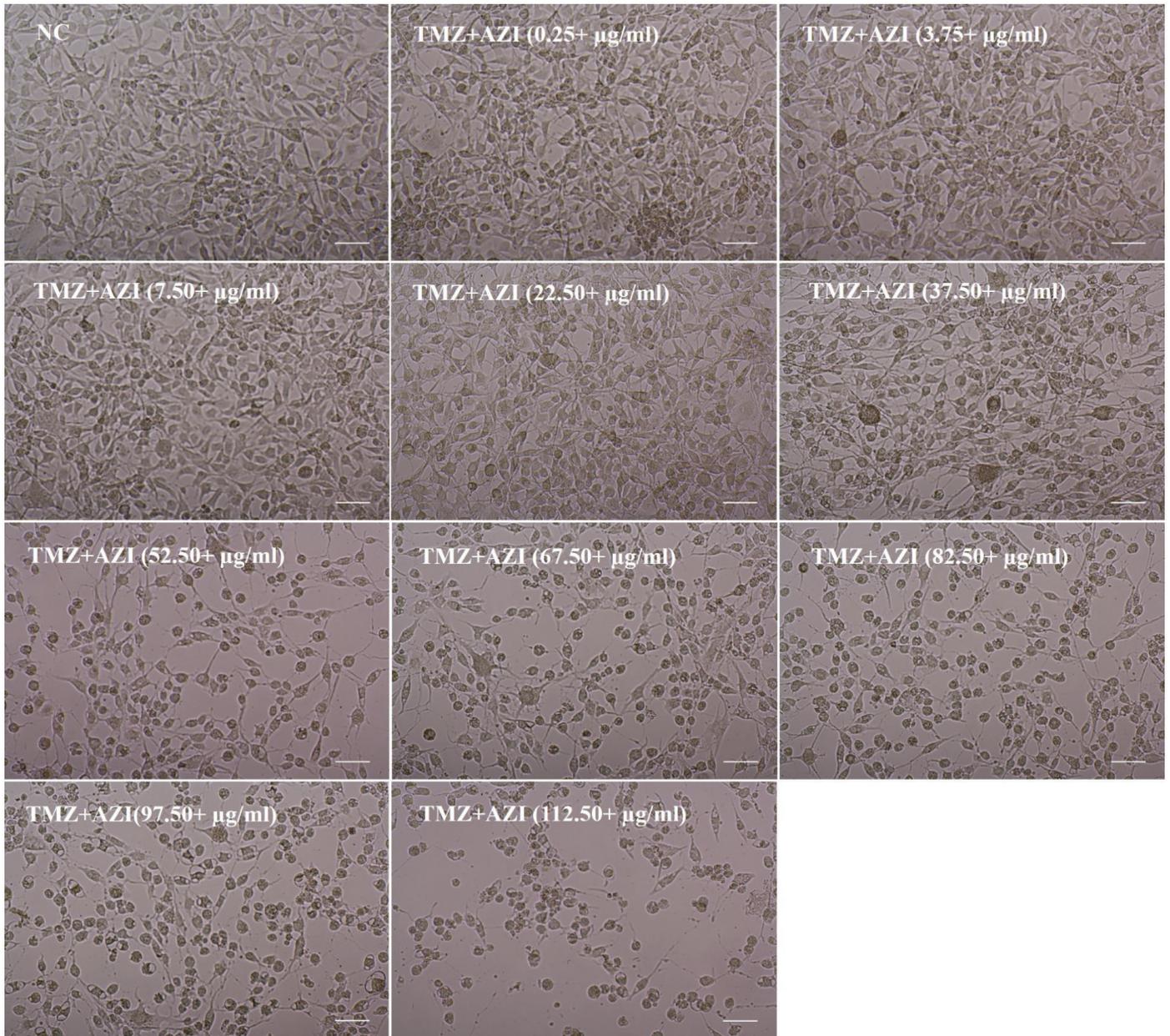


Figure 4. Effect of TMZ + AZI on U87 cells. Microphotographs were taken after 48h of incubation with different concentrations of TMZ + AZI, (scale bars = 100 µm).