Title: **PARP inhibitor counteracts Temozolomide Resistance in Glioblastoma Multiforme**

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Abbreviations:

TMZ: Temozolomide; GBM: Glioblastoma Multiforme; O6MeG: O6-methylguanine; N7MeG: N7-methylguanine; N3MeA: N3-methyladenine; MGMT: O6-Methylguanine-DNA-Methyl Transferase; BER: Base Excision Repair; PARP: Poly (ADP-Ribose) Polymerase; NHEJ: Non-Homologous End Joining; HR: Homology-directed Repair; OLA: Olaparib.

Abstract

Glioblastoma multiforme (GBM), a highly aggressive brain tumor, frequently develops resistance to temozolomide (TMZ), the current standard chemotherapy. Our study investigates the potential of combining TMZ with the poly (ADP-ribose) polymerase inhibitor Olaparib (OLA) to overcome TMZ resistance. Using *in vitro* models, including U251 cell lines and patient-derived GBM primary cultures, we demonstrate that OLA enhances TMZ efficacy by disrupting base excision repair and potentiating DNA damage-induced cytotoxicity. A CRISPR knockout screen identified DNA mismatch repair (MMR) gene deficiencies as key drivers of TMZ resistance, which OLA effectively counteracts. Co-treatment with TMZ and OLA significantly reduced tumor cell viability, even in MMR-deficient and MGMT-expressing contexts, suggesting a synergistic mechanism. Gene expression analysis revealed that the combination therapy impacts cell cycle regulation, stress responses, and extracellular matrix integrity, leading to mitotic catastrophe and apoptosis. These findings propose the TMZ-OLA combination as a promising therapeutic strategy for overcoming chemoresistance in GBM.

INTRODUCTION

Glioblastoma (GBM), a WHO classification 4 grade tumor, is the most common and most aggressive malignant brain tumor in adults (Thakkar et al. 2014; Louis et al. 2021). These highly proliferative gliomas mainly arise in the supratentorial brain (Oronsky et al. 2021) and are characterized histologically by high cellularity, high mitotic activity, vascular proliferation, and necrosis (Omuro and DeAngelis 2013). GBM display high inter and intra tumoral heterogeneity, which has been suggested to contribute to resistance to treatments (Inda et al. 2014). Indeed tumor cells may differ in terms of molecular, phenotypic, and functional characteristics, potentially enabling less sensitive tumor cell subpopulations to drive resistance to cancer drugs (Cloughesy et al. 2014).

Standard of care for GBM consists of surgical resection of the tumor if feasible, followed by a post-operative combination of concomitant radiotherapy and chemotherapy with Temozolomide (TMZ), known as “Stupp protocol” (Stupp et al. 2005). Due to its diffuse and infiltrative nature, as well as the risks of damaging intact brain tissue surrounding the tumor, complete surgical resection of GBM cannot be achieved in virtually all patients, making the use of complementary treatments mandatory (Oronsky et al. 2021). TMZ is a monofunctional alkylating agent which induces cytotoxic lesions including O6-methylguanine (O6MeG), N7-methylguanine (N7MeG) and N3-methyladenine (N3MeA) adducts (Gupta et al. 2018). The DNA mismatch repair pathway (MMR) pathway, comprised of several proteins (MSH2, MSH3, MSH6, MLH1 and PMS2), is essential to mediate cytotoxicity of TMZ: mispairing of O6MeG with another thymine leads to repetitive rounds of MMR, known as “futile cycling”, which finally induces double-strand breaks and triggers p53-dependent cell cycle arrest and apoptosis (Hickman and Samson 1999; Sarkaria et al. 2008). These lesions are repaired by the O6-Methylguanine-DNA-Methyl Transferase (MGMT) enzyme, expressed by approximately 40% GBM tumors, leading to resistance to TMZ (Esteller et al. 2000; Hegi et al. 2005; Wick et al. 2014). In the other GBM tumors the MGMT enzyme is however silenced by promoter methylation, resulting in persistent O6MeG lesions and potential cytotoxicity. On the other hand, N7MeG and N3MeA adducts, which account for approximately 90% methylation events induced by TMZ, are usually repaired by the base-excision repair (BER) pathway and contribute minimally to overall cytotoxicity of TMZ (Sarkaria et al. 2008; Gupta et al. 2018). Action of BER pathway is coordinated by the Poly (ADP-Ribose) Polymerase (PARP) family enzymes, which bind to the damaged DNA to recruit BER components (Curtin 2005; Helleday et al. 2008).

Despite aggressive treatment of GBM, comprising surgery and adjuvant radio-chemotherapy as evocated above, prognosis remains dismal with a median overall survival of 14 to 15 months and a five year survival rate of only 5% (Stupp et al. 2009; Ostrom et al. 2014). Almost all TMZ sensitive GBM become drug resistant over time, leading to tumor recurrence and clinical progression (Yuan et al. 2020). Unfortunately, treatments used for tumor recurrence such as chemotherapy or targeted therapy failed to show significative clinical efficacy and survival improvement (Oronsky et al. 2021). Development of new therapeutic modalities therefore seems essential to improve care for patients with GBM.

One mechanisms of acquired resistance to TMZ is the gain of mutations that inactivate the MMR pathway, thus inducing tolerance to DNA mispairing via O6MeG adducts, leading to cell survival (Yip et al. 2009; Johnson et al. 2014; Wang et al. 2016). A way to overcome alkylating chemotherapy failure could be a targeting of the BER pathway, which inactivation could increase cytotoxicity of N7MeG and N3MeA adducts, otherwise repaired in normal cells and tumor cells with an intact BER pathway (Bryant and Helleday 2004; Gupta et al. 2018). The use of small molecule inhibitors of the PARP enzymes has shown promising results in preclinical models (Donawho et al. 2007; Tentori et al. 2014; Yuan et al. 2020; Hwang et al. 2021a), through a potentiation of TMZ therapy by BER disrupting and other mechanisms as destabilization of replication forks (Murai et al. 2012a). Furthermore, it has been suggested that PARP inhibitors could also be effective for cancer cells expressing MGMT (without MGMT promoter methylation) (Erice et al. 2015a), due to the necessary regulation of MGMT by PARP for repairing TMZ-induced O6MeG adducts (Wu et al. 2021a).

PARP1-inhibitors (PARP1i) have been marketed with specific indications with respect to homologous recombination (HR) status, which is considered a predictive biomarker in response to PARP1i (Grignani *et al.*, 2020). In cells which have homologous repair deficiency (HRD), non-homologous end joining (NHEJ) would result from double strand break (DSB) formation, but with respect to homologous repair (HR), considered “error-free”, NHEJ affords DSBs repair with almost no consequence for sequence homology. The error-prone NHEJ promotes accumulation of DNA damage, and its activity is a major driver for PARP1i synthetic lethality in HR-defective cells (Patel *et al*., 2011).

We present the results of *in vitro* experiments studying the use of the inhibitor of the enzyme poly-(ADP-ribose) polymerase 1 (PARP1), Olaparib (OLA), in combination with TMZ in the treatment of GBM tumor cells. Experiments were done on U251 cell lines, which had either no prior treatment or had previously been made resistant to TMZ by prolonged exposure. Then, experiments were replicated on several patient derived cell lines.

RESULTS

Discovery of TMZ-resistance genes by CRISPR screen knock-out.

Based on our observations from Rabé *et al*. (2020), we first conducted a CRISPR screen knock-out to identify genes involved in TMZ resistance. We generated a Cas9-expressing U251 clone and transduced it with the Brunello lentiviral library (gift from David Root and John Doench; https://doi.org/10.1038/nbt.3437 PubMed 26780180). The cell library was expanded and treated with vehicle or with a therapeutic concentration of TMZ. About 95% of the cells died upon this TMZ insult. We harvested the cells once the surviving cells reached confluence after 18 days. After library preparation and sequencing of the sgRNA carried by the vehicle- and TMZ-treated cells, we analyzed the data using MAGeCKFlute (https://doi.org/10.1038/s41596-018-0113-7) and found gene knock-outs significantly enriched in the TMZ-treatment condition, suggesting that these gene invalidations would confer resistance against TMZ. We found several genes from the DNA mismatch repair (MMR) pathway: EXO1, MLH1, MSH2, MSH6, PMS1, PMS2. We also found 3 other invalidations: BCL11A, CLHC1 and GPR75 (**Figure 1A**). We did not find any genes whilst KO confers a higher sensitivity to the TMZ treatment.

We decided to proceed by validating the genes of interest by knock-down in U251 cells. We performed siRNA against the MMR members MSH2, MSH6, PMS2 and against BCL11A, CLHC1 and GPR75 (**Figure 1B**). Only the knock-down against the MMR members MSH2, MSH6 and PMS2 conferred resistance against TMZ. This result suggests that, in the context of loss-of-function mutations, MMR deficiency is the main driver of TMZ resistance in GBM.

Combination of the PARPi Olaparib and TMZ counteracts TMZ-resistance in U251 cells.

We tested the hypothesis that a Poly (ADP-ribose) polymerase inhibitor (PARPi) could counteract TMZ resistance driven by MMR deficiency.

We found the PARPi Olaparib (OLA) 1 µM having no effect on U251 cells in term of cell proliferation, viability and morphology even after 16 days culture. In contrast, TMZ induced about a 90% cell death until day 12 after which the cells recommenced to proliferate as shown in Rabé *et al*. (2020), while the cells treated with OLA plus TMZ showed a marked decrease in cell number and there was no increase in cell number even after 3 weeks (**Figure 2A-B**).

We have shown, that there is a selection of U251 cells resistant to TMZ after continual treatment with TMZ. These cells which we call U251-R cells were also treated with OLA and TMZ, and as shown in **Figure 2C-D,** we were able to induce greater cell death than with TMZ alone.

Olaparib seems to have a synergetic effect when combined with TMZ. We then questioned whether this was still true in a MMR-deficient context.

We knocked-down MSH6 in U251 cells and treated the cells with control, OLA-only, TMZ-only and TMZ plus OLA (**Figure 3A-B**). We confirmed that MSH6 knock-down confers a strong resistance against TMZ. However, when added to TMZ, the OLA strongly reduces cell growth in MSH6-depleted cells. These results suggest that TMZ and OLA have a synergetic effect that is able to counteract the TMZ-resistance induced by MSH6 deficiency.

Combination of the PARPi OLA and TMZ counteracts TMZ-resistance in glioblastoma primary culture.

We then tested the combination of TMZ plus OLA on glioblastoma primary culture at the time of diagnosis. We found synergetic effect of TMZ and OLA in several patients: GAIJa, G5, MERGi, QUECh, GONDo-R, A1-R (**Figure 4A-B** and **Supplementary Figures S1-7**). In most cases after 17 days, the TMZ plus OLA treatment was able to eradicate all cells while the TMZ-only failed to do so. For GAIJa patient, we also cultured its relapsed GBM surgical excision and treated it with TMZ-only, OLA-only or their combination. Again, the combination of TMZ and OLA has a stronger repressive effect on cell growth than TMZ alone (**Figure 4C-D B** and **Supplementary Figures S1-7**).

In GAIJa patient, at diagnosis and relapse, we could observe that the cells cannot undergo cytokinesis when treated with TMZ plus OLA. This suggests that this synergetic effect of TMZ plus OLA could be mediated by mitotic catastrophe (**Figure 5**).

RNAseq transcriptomic analysis

RNAseq data presented are for U251 cells treated with Temozolomide vs. U251 cells treated with TMZ + OLA. Results are presented in the form of an MA-Plot (Fig. 14A).

Thus, we have highlighted genes that are over-expressed (so-called “up-regulated”) and genes whose expression is diminished (so-called “down-regulated”) by dual therapy with TMZ + OLA compared with TMZ alone (**Fig. 6A-B**).

Various cellular processes seem to be affected:

1- Cell cycle disruption - cell division

- Down-regulation of TOX3, which keeps NHEJ active, prone to “serious” errors that do not allow passage to M phase at the G2/M checkpoint; down-regulation of AGAP, resulting in loss of PI3K activity and, ultimately, reduced HR activity.
- down-regulation of NINL and MAST1, involved in microtubule action, resulting in disruption/diminution of mitosis and hence cell division

2- Stress response

Up-regulation of IL11, CXCL8, SERPINE1, GDF15, involved in inflammatory responses and of GDF15, NGFR, CTGF, HBEGF whose expression increases in the event of cellular stress.

3- Extracellular matrix degradation

Up-regulation of MMP3, PLAU, CPA4, MGP, extracellular matrix degradation enzymes, and up-regulation of MSMP, EMP1, THBS1 and SERPINE 2, reflecting an attempt to adapt to the loss of cell cohesion.

Down-regulation of BOC involved in cell adhesion

4- Cellular metabolism

Down-regulation of SLC38A3, SLC29A4, transporters involved in amino acid and nucleoside metabolism, NOXA1 (reduced ROS production) and carbonic anhydrase IX, whose reduction affects adaptation to hypoxia and acidosis.

DISCUSSION

PARP inhibitor’s choice

Despite the nature of in vitro experiments, the choice of a PARP inhibitor was made with the idea of an in vivo translation, taking into account the existence of the blood brain barrier (BBB). Some promising PARP inhibitors have limited distribution across the blood brain barrier (Sarkaria et al. 2018). Talazoparib and rucaparib are certainly potent but are substrates for efflux transporters and/or breast cancer resistance protein (BRCA), which are active in brain endothelial cells (Parrish et al. 2015; Kizilbash et al. 2017). Due to their low penetration into the brain, these molecules have a limited distribution in the brain. On the other hand, olaparib and veliparib penetrate the BBB (Hanna et al. 2020).

Secondly, with regard to the choice of olaparib versus veliparib, we were interested in the direct effects of either PARP inhibitor on PARP1. PARP inhibitors have the ability to “PARP trap”. In this sense, the inhibitor molecule does not necessarily block PARP1 recruitment to the site

of DNA damage (equivalent to an inhibited catalytic activity), but it does block the effector cascade (Hopkins et al. 2019), so PARP-DNA binding is possible, but without activation of the rest of the repair systems.

This PARP trapping on DNA lesions has been shown to be more cytotoxic than the single-strand lesions themselves (Pommier et al. 2016).

The degree of PARP1 trapping varies between PARP inhibitors: olaparib >> veliparib (Hopkins et al. 2019).

There are two co-existing mecanisms :

- blocking PARylation, which reinforces PARP binding to DNA (Lindahl et al. 1995; Mortusewicz et al. 2007; Gagné et al. 2012) (406-408)
- binding of the therapeutic molecule to the NAD+ site, which increases the allosteric binding of PARP to DNA via its N-terminal zinc finger domain, as is the case with olaparib compared with veliparib (Murai et al. 2012a).

The allosteric enhancement of PARP-DNA binding by PARP inhibitors is probably more important for the anti-tumor activity of PARP inhibitors than catalytic inhibition itself.

Murai et al. have shown that PARP inhibitors, supposedly equivalent on the basis of catalytic inhibition, are not equivalent in terms of their anti-tumor activity, suggesting the importance of taking this allosteric binding enhancement data into consideration: depending on the PARP inhibitor used, clinical and experimental results can be very different. Their data explain why veliparib has a much lower toxicity than olaparib, despite these two molecules having a similar potency for PARP catalytic activity (Murai et al. 2012b).

Synergistic mecanisms

Concomitant treatment with TMZ and Olaparib demonstrated a synergistic effect of this combination, with greater cytotoxicity than with TMZ alone.

1- Cycle cell :

We assume that the effect of a PARP inhibitor is not immediately manifested by an increase in cell death, due to the much longer average time a tumor cell has before replicating. The potential cytotoxicity of DNA lesions occurs at the end of S phase, at the beginning of G2 phase (check-

points of duplicated DNA integrity) (Liu et al. 1999). DNA quantification analyses (NucleoCounter®) carried out on GAIJA primo-cultures revealed a higher proportion of S-phase cells (or aneuploid cells) in Olaparib alone condition or co-treatment with TMZ and Olaparib than in untreated cells or cells treated with TMZ alone. Finally, more dead cells (apoptosis and mitotic catastrophe) were noted in the co-treatment condition, for both primo-cultures, but in higher proportion for $GAIJA_R$ compared to $GAIJA_P$. This phenomenon, albeit less severe, was noted in the Olaparib-only condition. This would imply that tumor cells, over time, under the pressure of TMZ treatment, accumulate DNA lesions, with a certain tolerance, remain stuck in S phase, without division, and the action of a PARP inhibitor unmasks the alkylating lesions, with passage into G2/M phases and committing tumor cells to apoptotic cell death.

This also echoes RNAseq data with down-regulation of TOX3 and AGAP2.

Our results confirmed the presence of death by apoptosis (red) and by mitotic catastrophe (green) without clusters of tolerant cells (**Fig. 5**).

This effect of apoptotic rather than necrotic cell death has also been reported in the literature (Hwang et al. 2021a).

2- MGMT

The use of a PARP inhibitor makes it possible to act on both levels of TMZ alkylation:

- preventing/disrupting PARylation of MGMT and enabling/increasing TMZ cytotoxicity via 06MeGs (Wu et al. 2021b).
- prevent/disrupt BER activation and induce TMZ cytotoxicity via N7MeG and N3MeA.

Among our co-treated cells, MGMT status was known:

- U251 lines: natural methylation of the MGMT promoter
- U251-R lines: according to previous datas (Rabé et al. 2020), de novo expression of MGMT
- Primo-cultures: unmethylated MGMT's promoter

The relative homogeneity of response to dual therapy with TMZ + OLA between the U251 (non-functional MGMT) and U251-R (de novo functional MGMT) lines could therefore be explained by the PARylation blocking effect of Olaparib, which does not over-activate MGMT and provides equivalent cytotoxicity of MGMT-expressing cells to that seen in cells with non-functional MGMT. The PARP inhibitor seems to have a "catch-up" action on active-MGMT tumour cells. Some authors have demonstrated the sensitizing effect of PARP inhibitors on

Temozolomide-resistant cell lines, particularly in melanoma, colorectal cancer and glioblastoma. Indeed, PARP inhibitors showed sensitizing effects only on cell lines overexpressing MGMT, in contrast to cell lines that do not express MGMT through promoter methylation (Erice et al. 2015b; Gupta et al. 2016). We have also observed this “catch-up” phenomenon.

Our results suggest that PARP inhibitors may also enhance the effect of TMZ on tumor cells with functional MGMT, which is another advantage of using PARP inhibitors against glioblastoma.

3- BER/HR/NHEJ systems

In order to better elucidate the mechanisms involved, particularly in relation to MMR inactivation, and with a view to validating the potential efficacy of TMZ + OLA co-treatment on GBM that have acquired resistance to TMZ, we treated U251KO-MMR cells with either single or dual therapy. We first demonstrated that MMR knockout lead to TMZ resistance. In contrast, co-treatment with TMZ and OLA induced significant cytotoxicity, bypassing the MMR system, the main mechanism of TMZ resistance. This raises the hypothesis that the PARP inhibitor blocks the BER system, thereby enhancing TMZ cytotoxicity, a concept reported in several publications (Hwang et al. 2021b; Wu et al. 2021a).

However, the literature is not unanimous and the involvement of double-stranded lesions in mediating cytotoxicity should be considered:

- Some authors noted, in an MMR-KO model (MSH6-KO) with complementary co-inactivation of the BER system, persistent cytotoxicity of co-treatment with TMZ and Veliparib (Higuchi et al. 2020). We might have expected a restoration of TMZ resistance, highlighting the central role of the BER system.
- Others showed no accumulation of single-stranded lesions, despite the use of a PARP inhibitor blocking the BER system. According to these authors, single-stranded lesions are converted into double-stranded lesions (Helleday 2011; Ström et al. 2011).

PARP inhibitors were initially marketed with specific indications concerning the status of homologous recombination (HR), notably BRCA mutations, which are considered predictive biomarkers of response to PARP inhibitors (Grignani et al. 2020). Tumours with impaired homologous recombination are directed towards another repair alternative, the NHEJ. NHEJ, which is naturally error-prone, favours the accumulation of DNA damage, increasing aggressiveness and tumour invasion through sub-optimal repair (Jackson and Bartek 2009).

However, PARP inhibitors alter the precision of NHEJ, leading to aberrant repair and apoptosis, and their activity is therefore one of the main factors in the lethality of cells defective in homologous repair (Patel et al. 2011).

It would seem, then, that the efficacy of PARP inhibitors relies more on alteration of NHEJ than on inactivation of BER, which nonetheless, by delaying its activity, contributes to the increase in double-strand breaks processed by HR and NHEJ.

Interestingly, when homologous recombination is functional (partially or fully), PARP proteins are able to inhibit the binding of proteins involved in NHEJ promotion to double-strand break sites. This facilitates the recruitment of homologous recombination (Hurley et al. 2019). Although PARP inhibition, even in cells with functional HR status, would allow DNA repair to be directed towards NHEJ, the addition of HR inhibitory treatment could potentiate the cytotoxic effect of PARP inhibitors.

We know that GBM do not have BRCA mutations. The expression of HR (via BRCA1/2) depends on the activity of PI3Kα (González-Billalabeitia et al. 2014), which is over-activated in glioblastomas (Parsons et al. 2008). Consequently, PI3K inhibitors would inactivate HR in GBM. (Zhang et al. 2021) confirmed that a PI3K inhibitor (BKM120) induced HR deficiency and sensitized GBM to PARP inhibitors, opening up new perspectives in the treatment of GBMs.

However, PI3K inhibitors are currently undergoing clinical trials (Atkins et al. 2015), and their toxicity has been raised (Konstantinopoulos et al. 2019).

Some authors studied, in vitro and in vivo (murine xenografts), the efficacy of a PARP inhibitor (Niraparib) combined with a topoisomerase I inhibitor. Synergistic cytotoxicity was demonstrated, with HR blockade on PTEN-altered GBM cells and significantly longer survival of xenografted and treated mice (Kim et al. 2023).

This raises two questions:

- Is PTEN status predictive of response to PARP inhibitors?
- Could the combination of treatments, in particular PARP and PI3K inhibitors be more effective?

MATERIALS AND METHODS

Materials: Temozolomide (LSC711) and Olaparib were obtained from Interchim (Montluçon, France), all other drugs used in this study were from Sigma (Saint Louis, MO). All cell culture products were obtained from Life Technologies (Carlsbad, CA).

Cell culture: After informed consent, tumor samples classified as Glioblastoma type IV (GBM) based on WHO criteria, were obtained from patients undergoing surgical intervention at the "CHU de Nantes" and the "Tumorothèque IRCNA". Within 4 h after surgical removal, GBM cells were recuperated after mechanical dissociation as described in *Brocard et al.* (2015). All procedures involving human participants were in accordance with the ethical standards of the ethic national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. GBM cells were cultured in defined medium (DMEM/F12 supplemented with 2 mM L-glutamine, N2 and B27 supplement, 2 µg/ml heparin, 20 ng/ml EGF, 40 ng/ml bFGF, 100 U/ml penicillin and 100 μg/ml streptomycin). All the experiments with primary GBM cells were performed at early passages. Cells were analyzed for mycoplasma regularly.

U251 cells were cultured in DMEM (Gibco, ref. 21969035) enriched with 10% FCS with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. U251 cell line was certified by Eurofins Genomics (Ebersberg, Germany). Cells were cultured at 37°C with 5% CO_2 and 95% humidity. The cell medium is replaced every 2-3 days with fresh medium and the cells are reseeded in a new dish when the cell layer was approximately 80% confluent.

CRISPR KO screen (cell culture). We generated Cas9-expressing U251 clones by lentiviral transduction (Addgene, ref. 52962-LV) and blasticidine selection. We checked Cas9 expression by Western-Blot and selected the clone with the most efficient Cas9 nuclease activity using a Cas9 activity reporter (Addgene, ref. 67983 and ref. 67984). The Cas9-expressing U251 clone was expanded, transduced with the Brunello lentiviral library at MOI 0.3 (gift from David Root and John Doench; Addgene, ref. 73178-LV ; doi: 10.1038/nbt.3437. PubMed 26780180) and selected with puromycin. After expansion to 3 x 10^8 cells: 10^8 cells were harvested and frozen down, 10^8 cells were treated with 50 µM TMZ and 10^8 cells were treated with DMSO (vehicle condition). For the TMZ-treated cells, the medium was changed every 3 days with fresh TMZ. A minimum of $100x10^6$ DMSO-treated cells were replated every 3 days in fresh medium. The cells were cultured during 18 days, until the TMZ-treated cells reached the sub-confluence. At this point, DMSO-treated cells and TMZ-treated cells were harvested and frozen down.

CRISPR KO screen (library preparation). Genomic DNA was purified using Qiagen Blood & Cell Culture DNA Maxi Kit (ref. 13362). SgRNA sequences were amplified by PCR on genomic DNA using Q5 (NEB, ref. M0492S) and P5/P7 primers as recommended by Doench JG and colleagues (doi: 10.1038/nbt.3437. PubMed 26780180). Illumina libraries were quality-controlled by Agilent TapeStation, quantified by qPCR and submitted for HiSeq sequencing at the genomics core facility GenoA in Nantes, France.

PARP1 inhibitor treatment. The PARP1 inhibitor, Olaparib (OLA) was dissolved in DMSO to give a stock concentration of 10 mM. In all experiments, the final working concentration of OLA was 1 µM. The medium in the dishes was replaced every 2-3 days. DMSO was used as a vehicle control in all experiments (**Fig. S8**).

Infection of U251 cells with siRNA. Cells were plated at 10^5 cells/well in 6-well plates and 24h later transfected with 600 ng/ml siLuc, siMSH2, siMSH6 or siPMS2 (MISSION® esiRNA, Sigma-Aldrich, Saint-Louis, MI) using the using the Lipofectamine RNAiMax transfection reagent (Thermo-Fisher Scientific, Waltham, MA) for 48h and then used in experiments.
RGB cells: The human U251 cell line LeGO-RGB lentiviral vectors were used as color-guided clonal cell trackers. LeGO-C2 (Addgene: 27339), LeGO-V2 (Addgene: 27340) and LeGO-Cer2 (Addgene: 27388) plasmids were employed to produce lentiviral vectors coding for red, green and blue fluorescent proteins, respectively according the method previously described in Gambera et al. (2018). The RGB color mix was achieved using a MOI of 0.75, which corresponds to an equimolar transduction efficiency of 50% per lentiviral vector 3 days after transduction. Cell transduction efficacy and colored cell distribution and stability were followed by FACS analyses.

FACS analysis: The cells were trypsinized, counted and then resuspended in 1 ml PBS containing 1% BSA. Data acquisition was performed with the BD FACSymphony (BD Biosciences®) and BD FACSDiva 8.5 analysis software. The cerulean protein was excited at 405 nm and detected with a 525/50 filter. The venus protein was excited at 488 nm and detected with a 530/30 filter and the cherry protein was excited at 561 nm and detected with a 610/20 filter. The cells were initially sorted according to their size (Forward Scatter, FSC) and their complexity (Side Scatter, SSC). The U251-RGB cell population was selected and doublets were excluded. Using three different strategies namely; a green strategy allows for the visualization in a dot plot containing the blue, red and magenta cells; a red strategy allows for the

visualization in a dot plot containing the blue, green and cyan cells and a blue strategy allows for the visualization of red, green and yellow cells. The amount of unlabeled ($R^-G^-B^-$), red ($R^+G^-B^-$), blue ($R^-G^-B^+$), green ($R^-G^+B^-$), magenta ($R^+G^-B^+$), yellow ($R^+G^+B^-$), cyan ($R^-G^+B^+$), and white ($R^+G^+B^+$) cells were then quantified. The average number of events of each color was calculated and then transcribed as a percentage.

Microscopy: Photos of the cells were taken using an Axio Observer optical microscope (Zeiss®, Iéna, Germany) at 10x magnification. The images were then processed using ImageJ2 and Adobe Photoshop 8.0 software.

Gene expression assay: RNA reverse transcription was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo-Fisher Scientific, MA, USA). qPCR assay was performed in triplicates using the PerfectaTM SYBR® Green FastMixTM, Low ROXTM (Quanta BioSciences, CA, USA) and real-time thermal cycler qTower (Analytik Jena AG, Germany). Fold change was calculated using RPLPO, TATA, and HGPRT as housekeeping genes (n = 5).

Statistical analyses: Statistical analyzes were performed using one-way and two-way ANOVA tests with Prism 8 software. A p-value less than 0.05 is considered significant. The p-value of significant values is indicated by * < 0.05, ** < 0.01 and *** < 0.001.

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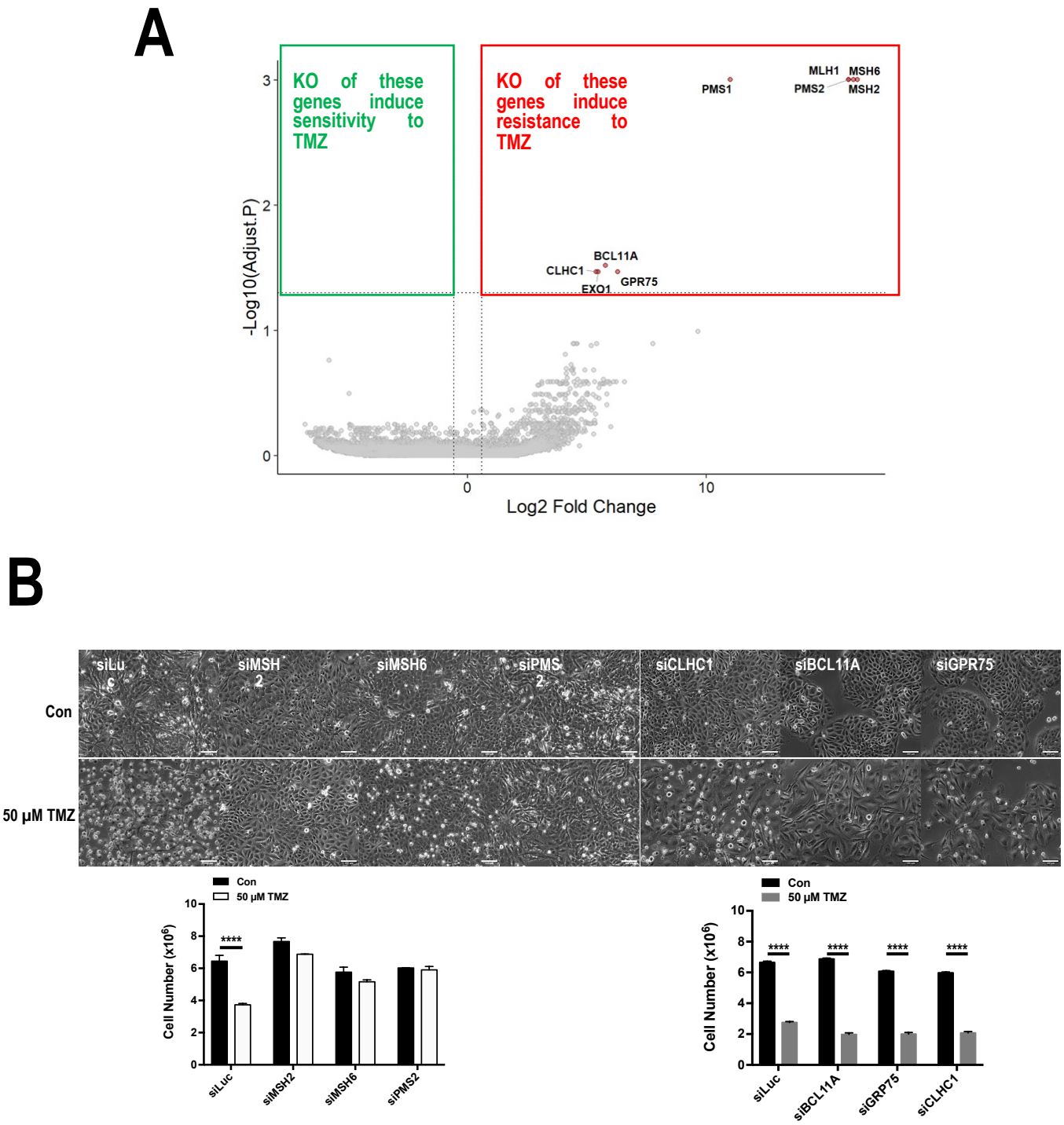
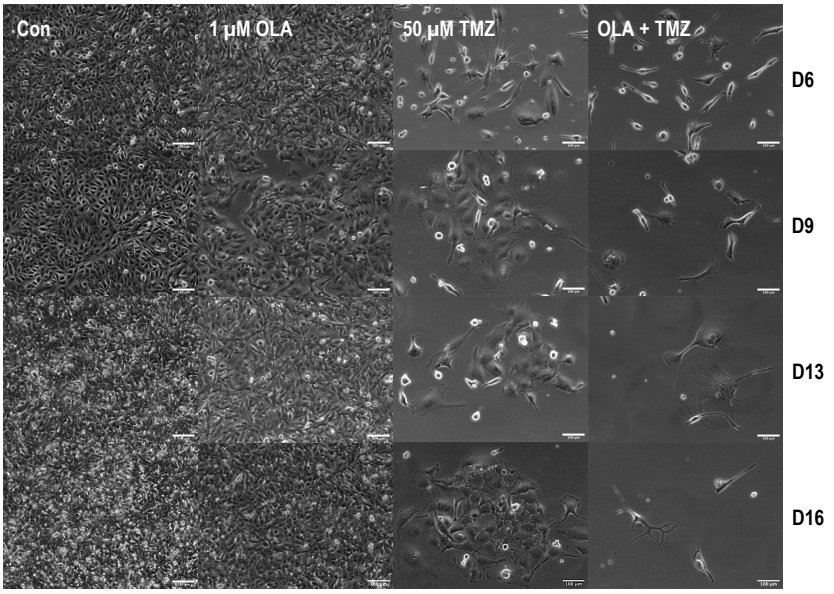

A
KO of these genes induce sensitivity to TMZ
KO of these genes induce resistance to TMZ
PMS1
MLH1
MSH6
PMS2
MSH2
BCL11A
CLHC1
GPR75
EXO1
-Log10(Adjust.P)
Log2 Fold Change
B
siLuc
siMSH2
siMSH6
siPMS2
siCLHC1
siBCL11A
siGPR75
Con
50 μM TMZ
Cell Number (x10^6)
****
siLuc
siMSH2
siMSH6
siPMS2
siBCL11A
siGRP75
siCLHC1

Figure 1

A

U251



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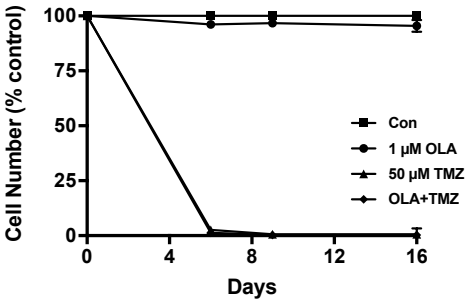
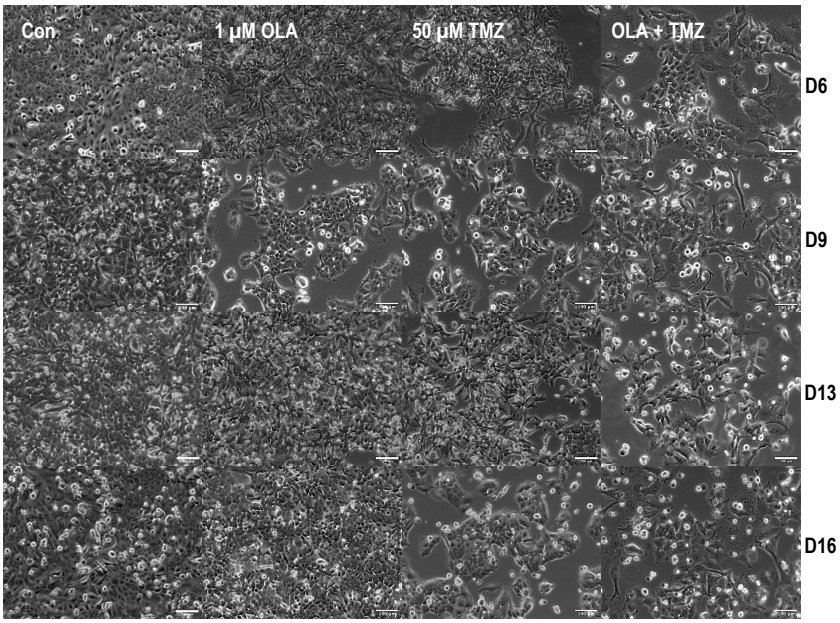


C

U251-R



D

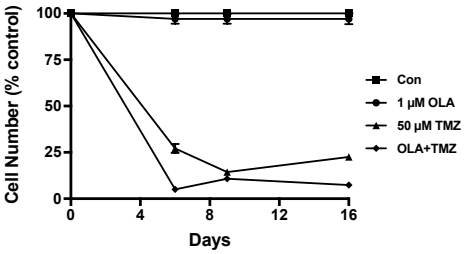


Figure 2

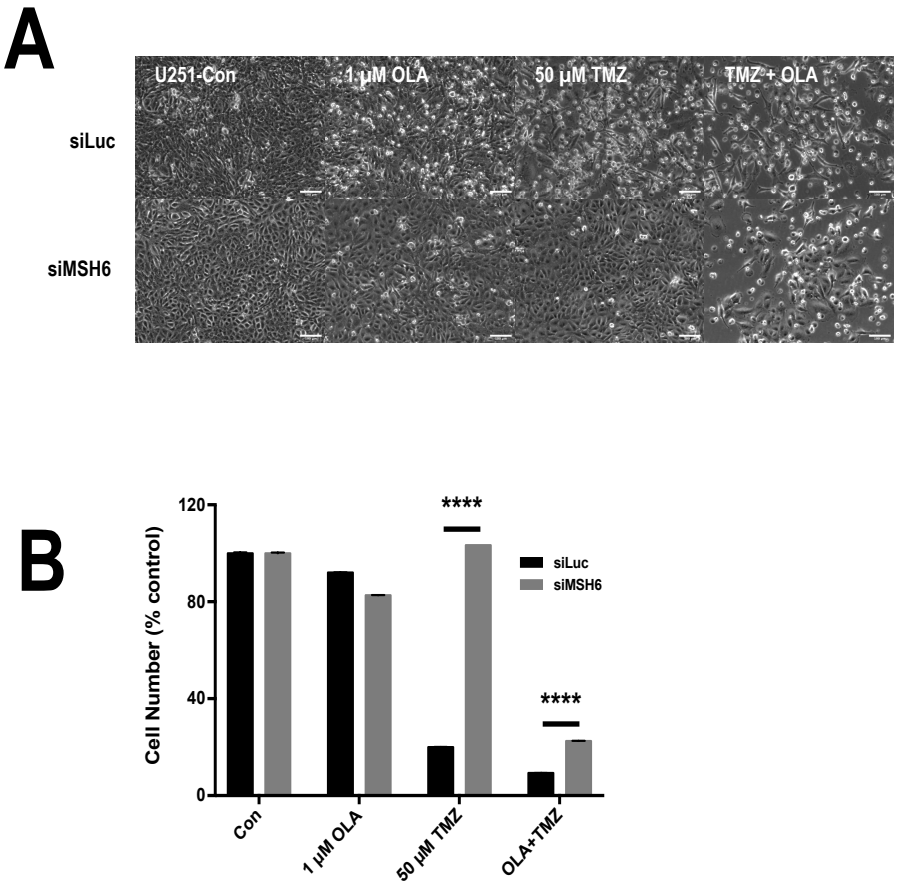
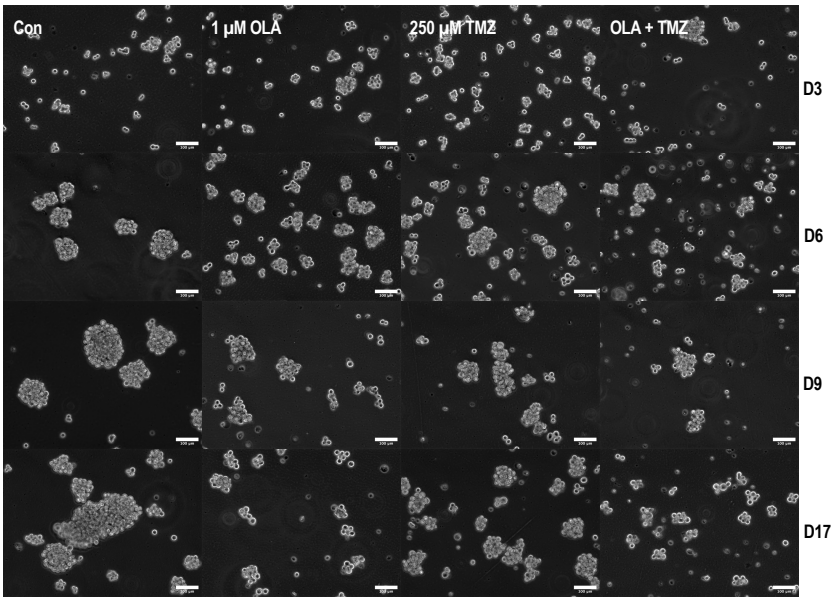

A
U251-Con
1 μM OLA
50 μM TMZ
TMZ + OLA
siLuc
siMSH6
B
Cell Number (% control)
120
80
40
0
****
****
siLuc
siMSH6
Con
1 μM OLA
50 μM TMZ
OLA+TMZ

Figure 3

A

GAIJa-Diagnosis



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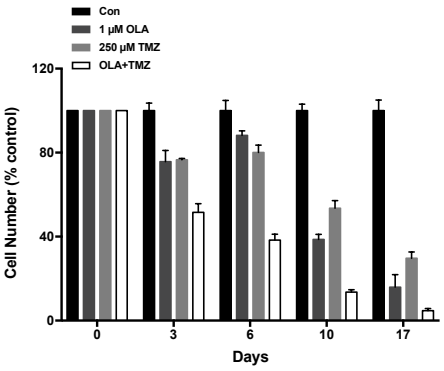
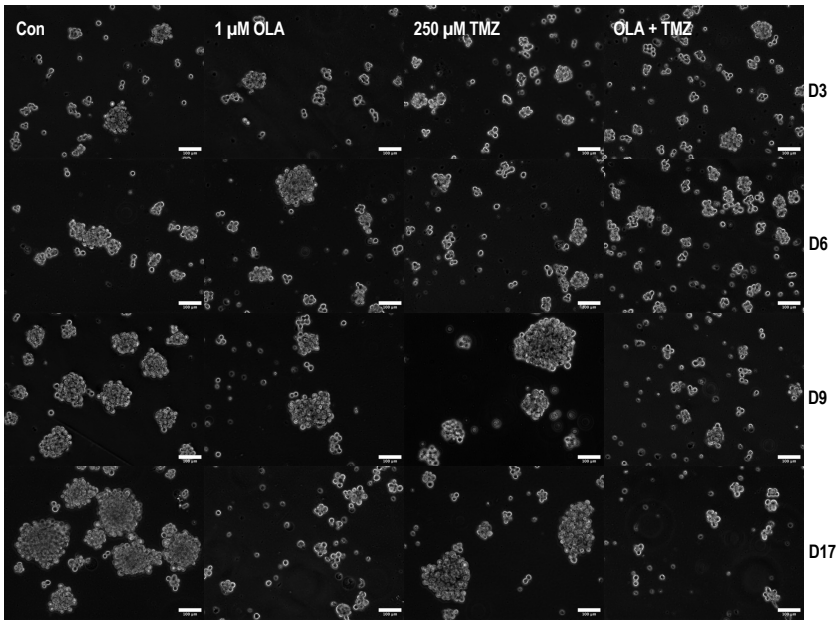


C

GAIJa-Relapse



D

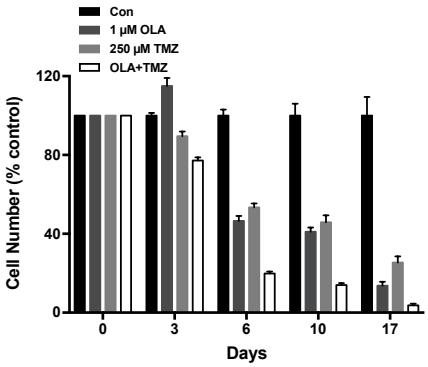
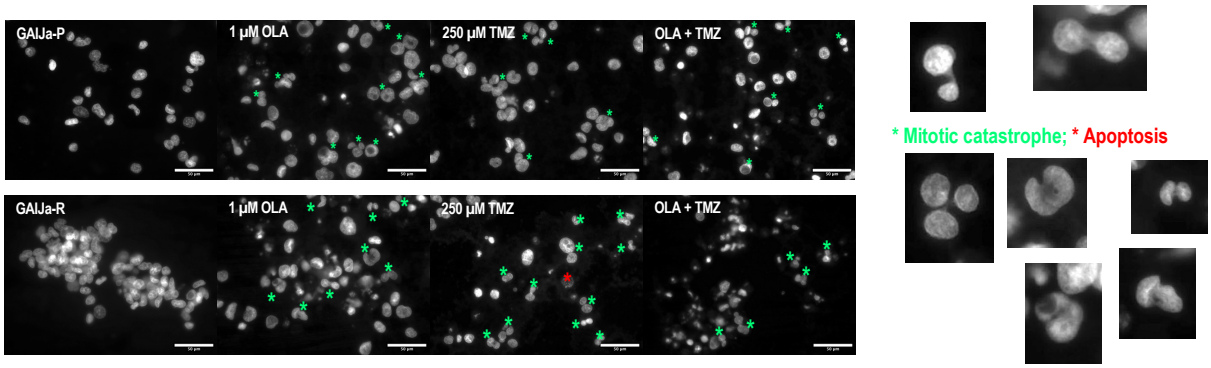
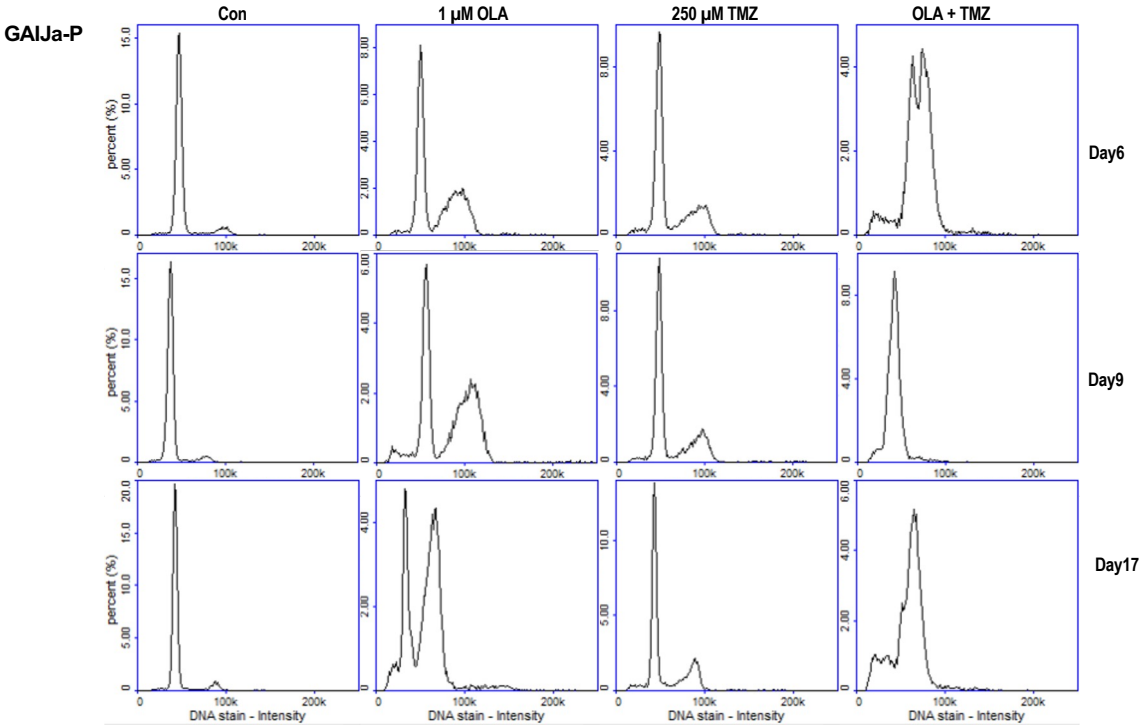


Figure 4


GAIJa-P
1 μM OLA
250 μM TMZ
OLA + TMZ
GAIJa-R
1 μM OLA
250 μM TMZ
OLA + TMZ
* Mitotic catastrophe; * Apoptosis


GAIJa-P
Con
1 μM OLA
250 μM TMZ
OLA + TMZ
Day6
Day9
Day17
percent (%)
DNA stain - Intensity

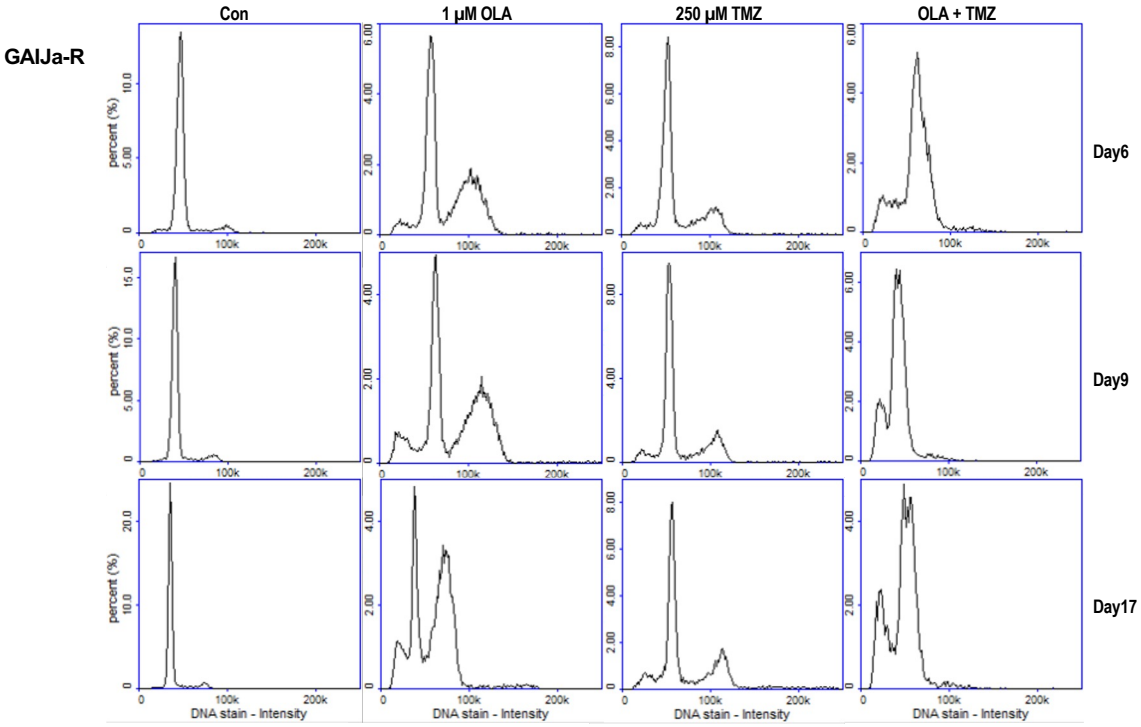

GAIJa-R
Con
1 μM OLA
250 μM TMZ
OLA + TMZ
Day6
Day9
Day17
percent (%)
DNA stain - Intensity

Figure 5