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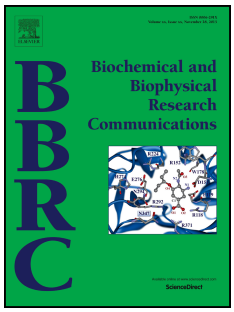

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The Combination of A-966492 and Topotecan for Effective Radiosensitization on Glioblastoma Spheroids

Fereshteh Koosha[a,b], Ali Neshasteh-Riz[b,d], Abbas Takavar[a], Nazila Eyvazzadeh[c], Zohreh Mazaheri[e], Samira Eynali[b], Mehdi Mousavi [f]

[a] Department of Medical Physics and Biomedical Engineering, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

[b] Radiation Biology Research Center, Iran University of Medical Sciences,

Tehran, Iran

[c] Radiation Research Center, Faculty of Para medicine, AJA University of Medical Sciences, Tehran, Iran

[d] Department of Radiation Sciences, School of Para medicine, Iran University of Medical Sciences, Tehran, Iran

[e] Department of Anatomical sciences, Medical Sciences Faculty, Tarbiat Modares University, Tehran, Iran

[f] School of Medicine, Jiroft University of Medical Sciences, Jiroft, Iran

Corresponding Author: **Ali Neshasteh-Riz**

Address: Department of Radiation Sciences, School of Para medicine, Iran University of Medical Sciences, Tehran, Iran, P.O. Box 1449614535, Shahid Hemmat Highway, Tehran, Iran

Email: neshastehriz@yahoo.com

Tel: +98-2188602218, Fax: 98-2188602218

Co-corresponding Author: **Abbas Takavar**

Address: 16 Azar street, Keshavarz boulevard, Department of Medical Physics and Biomedical Engineering, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Email: Takavar@tums.ac.ir

Tel: +98-2166466383, Fax: +98-2188973653

Abstract

Radiotherapy is one of the modalities in the treatment of glioblastoma patients, but glioma tumors are resistant to radiation and also chemotherapy drugs. Thus, researchers are investigating drugs which have radiosensitization capabilities in order to improve radiotherapy. PARP enzymes and topoisomerase I enzymes have a critical role in repairing DNA damage in tumor cells. Thus, inhibiting activity of these enzymes helps stop DNA damage repair and increase DSB lethal damages. In the current study, we investigated the combination of TPT as a topoisomerase I inhibitor, and A-966492 as a novel PARP inhibitor for further radiosensitization. U87MG cells (a human glioblastoma cell line) were cultured in Poly-Hema coated flasks to reach 300µm-diameter spheroids. Treatments were accomplished by using non-toxic concentrations of A-966492 and Topotecan. The surviving fraction of treated cells was determined by clonogenic assay after treatment with drugs and 6MV X-ray. The γ-H2AX expression was measured by an immunofluorescence staining method to examine the influence of A-966492, TPT and radiation on the induction of double stranded DNA breaks. Treatments using the A-966492 drug were conducted in concentration of 1µM. Combining A-966492 and TPT with radiation yielded enhanced cell killing, as demonstrated by a sensitizer enhancement ratio at 50% survival (SER_{50}) 1.39 and 1.16 respectively. Radio- and chemo-sensitization was further enhanced when A-966492 was combined with both X-ray and TPT, with SER_{50} of 1.53. Also γ-H2AX expression was higher in the group treated with a combination of drugs and radiation.

A-966492 is an effective PARP inhibitor and has significant radio-sensitivity on U87MG spheroids. By accumulating cells in the S phase and by inhibiting the DNA damage repair, TPT enhanced radio-sensitivity**.** A-966492 combined with TPT as a topoisomerase I inhibitor had additive radio-sensitizing effects. As a result, applying PARP and topoisomerase I inhibitors can be a suitable strategy for improving radiotherapy in clinics.

Key word: Glioblastoma, PARP inhibitor, Topoisomerase I inhibitor, Topotecan, Radiosensitivity

1. Introduction

Glioblastoma multiform (GBM) is the most aggressive and malignant glial tumors. Despite multidisciplinary methods in treating glioblastoma and different therapeutic modalities, patients with glioblastoma are faced with a poor prognosis [1] and the median survival following diagnosis is 12 to 15 months [2]. Aggressive surgery, chemotherapy with Temozolomide and radiotherapy are common methods in treatment of glioblastoma patients. The negative side-effects of radiotherapy have been observed in the treatment of high and low grade gliomas [3, 4]. Resistance of this tumor to current treatment modalities has encouraged scientists further investigate the effects of recent novel and nontoxic radio-sensitizing drugs in order to increase cell deaths via inducing more DNA damage in glioblastoma tumors. In radiotherapy, late radiation toxicity is irreversible and may increase with radiation dose [1, 5]; therefore, elevating the radiation dose with nontoxic doses of radio-sensitizing drugs could lead to less severe side effects and finally a more successful treatment. One of the most important targets of ionizing radiation in mammalian cells is DNA [6]. Interaction of ionizing radiation with DNA leads to Single Stranded Breaks (SSB) and Double Stranded Breaks (DSB). SSB induced by megavoltage X-ray is not cytotoxic [7]; however, unrepaired DSBs potentially lead to cell death. DSB may be repaired by Homologous Recombination (HR) or Non-Homologous Recombination Pathways (NHEJ). Poly (ADP) Ribose Polymerase Proteins such as PARP1 and PARP2 are key enzymes in repairing SSB as intermediate products of base excision repair (BER**)**, rapidly binding to SSB, and therefore, activating the repair process in cells [8]. Hence, inhibiting PARP1 and PARP2 proteins may lead to unrepaired SSB, which in turn may give rise to a potentially lethal DSB, resulting in the radio-sensitizing effect, specifically in replicating cells [9].

A-966492 is a novel PARP1 and PARP2 inhibitor, orally bioavailable and has special pharmacologic characteristics developed by Penning et al. [10]. A-966492 has the ability to cross the blood-brain barrier thus can be distributed into the tumor tissue. As the blood-brain barrier is one of the important problems in delivering drugs to glioblastoma tumors, our hypothesis states that applying A-966492 might be effective as the radio-sensitizing drug in treating glioblastoma.

Topotecan (TPT) is derived from Campothecin and is a potent anti-tumor drug used clinically and also in current preclinical studies [11]. TPT is a topoisomerase I inhibitor. After the formation of SSB, topoisomerase I covalently binds to 3′ end of the DNA and forms the Topo I-DNA cleavable complex. Topo I inhibitors, such as TPT stabilize this complex and extend the life time of the DNA strand break; consequently, the repair process becomes unsuccessful. And eventually, there is an increase in the formation of lethal DSBs

and cell death. Due to these characteristics, TPT is used in the therapy of different tumors including high grade glioma [12].

Human tumor environments are complex and heterogeneous, for example, tumors with diameters larger than 200µm have diverse types of cells including necrotic, hypoxic and normoxic which react differently towards radiotherapy and chemotherapy. multicellular tumor spheroids have greater chemotherapeutic resistance than the same cells in monolayer culture [13**].** Therefore, results from invitro experiments using spheroids as 3D cell culture models may have much more similarity to real tumors in comparison with 2D cell culture models in response to chemotherapy drugs and radiation [14]. Thus, in this study we used 300µm spheroids of U87MG cell line for our treatments. The purpose of this study is to examine the radio-sensitizing effects of A-966492 as a PARP1 inhibitor to 6MV X-ray and TPT as a Topoisomerase I inhibitor on glioblastoma spheroids.

2. Material and Method

2.1. Cell Line

The human GBM cell line U87MG was obtained from Pasteur Institute (Tehran, Iran). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, USA) with 10% fetal bovine serum (FBS; Gibco/Invitrogen, USA) and penicillin streptomycin (GmbH/PAA, Austria) at 37 °C in a humidified atmosphere containing 5% CO2 and 95% air. Spheroids were cultured using the liquid overlay technique. First, we coated T25 flasks with Poly-HEMA [15] (Sigma/Aldrich, Germany).
A total of 5×10^5 cells were seeded into a T25 coated flask with a thin layer of Poly-Hema with 5 ml of MEM supplemented with 10% FBS. The plates were incubated at 37˚C in a humidified atmosphere with 5% CO2 (Memmert, Germany) for 19 days[16], until they reach to approximately 300 µm in diameter. Half of the cultured medium was replaced with a fresh medium twice a week.

*2.2.*Drug Treatment and Irradiation

Glioblastoma spheroids were grown to reach a diameter of 300µm, then, they were treated with single drug and drug combinations. A-966492 (Selleckchem, USA) in solid powder form Diluted in Dimethyl Sulfoxide (DMSO) (Sigma), to a final concentration not exceeding 0.1% (at this concentration DMSO alone had no effect on cell viability). Topotecan (Hycamtin) was provided by Glasko Smith Kline (UK). 1mM stock solution was prepared, dissolving the powder in water. Samples were incubated with 1µM of A-966492 for 1 hour, and 1µM of Topotecan for 2 hours. Irradiation with different doses was performed using a linear accelerator (Linac 600, GMV; Varian Medical Systems; USA); at a dose rate of 2 Gy/min field size of 35*30 cm^2 and these flasks were irradiated from the posterior [16].

2.3. Colony-Formation Assay

Treated cells were seeded into 60 mm dishes, at various densities depending on the physical and chemical dose that cells received, and cultured in 5 ml serum-containing culture medium. Cells were incubated at 37˚C in a 5% CO2 incubator. After 12-15 days, the colonies were washed with free Phosphate Buffered Saline (PBS), and fixed with 5% formaldehyde and stained with 0.02% crystal violet solution. A cell cluster containing at least 50 cells was considered a colony. The surviving fraction was calculated as the number of colonies of treated cells divided by that for the control cells.

The X-ray dose-survival curves were fitted to the linear quadratic equation, surviving fraction $= \exp(-\alpha D - \beta D^2)$, where D is the X-ray dose. The Sensitizer Enhancement Ratio (SER) was used to evaluate the drug-radiation interaction. Its value was estimated by fitting it into the Linear-Quadratic (LQ) model as follows:

$SER_{x\%}=\frac{dx\%(no\ drug)}{dx\%(drug)}$ where $d_{x\%}$ (no drug) is the radiation dose (Gy) required to produce x% cell survival without drugs, and $d_{x\%}$ (drug) in the presence of drugs (i.e. TPT and/or A-966492). SER was calculated at doses related to surviving fractions of 10% and 50%.

2.4. Immunofluorescence staining

After irradiation and drug treatment, the spheroids were washed with 1ml PBS followed by adding 2 normal HCL, they were kept in this condition for 20 minutes before being washed with borate buffer. Next, these spheroids were permeabilized with Triton X-100 and goat serum for 45 minutes. They were then treated with primary antibody Anti-gamma H2A.X phospho S139 antibody (1:1000 Abcam, UK), and kept in the refrigerator. The cells were treated with 100µl secondary antibody, FITC, in the dark, stored at 37˚C for 2 hours and later washed with PBS. These cells were later, transferred to glass slides, Nuclei were stained with propidium iodide (PI). Then they were examined using fluorescence microscope (Nikon, Japan), and the green intensity of the phosphor-H2AX signal on digitized images was automatically analyzed using Image-J software.

2.5. Statistical Analysis

All experiments were either duplicated or triplicated. The error bars represent the standard error among the different experiments.

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Scheffe test for post-hoc analysis via SPSS software version 16. A significant level of $P < 0.05$ was

used for all analysis.

3. Results

3.2. Effects of Drug Treatments Combined with Radiation on Colony Forming

Cell survival was investigated after combined treatment with A-966492, TPT and X-ray on 300µm glioblastoma spheroids (Figure 1). As it is shown in Figure 1(A, B), TPT and A-966492 individually enhanced radio-sensitivity in U87MG spheroids compared to radiation alone. Figure 1D demonstrates that the survival fraction is significantly lower in the group treated with A-966492 compared to the group treated with TPT, indicating that A-966492 had a better radio-sensitizing effect. Also, the combination of TPT, A-966492 and X-ray significantly reduced the surviving fraction ($p<0.05$) in comparison with radiation alone. Table 1 shows the values of α and β in the linear quadratic equation for calculating the surviving fraction$= \exp(-\alpha D - \beta D^2)$, where D is the X-ray dose. The SER_{50} values were calculated for cells treated with TPT, A-966492 and the combination of these drugs in the presence of 6 MV X-ray. The combination of X-ray and A-966492 led to substantial radio-sensitizing effects with SER_{50}=1.39 which is significantly higher than SER_{50} of TPT accompanied by X-ray (1.16). This confirms that A-966492 is an effective radio-sensitizer of the U87MG cell line. The triple combination of X-ray, TPT and A-966492 had the strongest effect compared to any single agent with SER_{50} of 1.53 since A-966492 increased sensitivity to both radiation and TPT.

3.4. Determining γH2AX Formation after Irradiation and Drugs Treatments

In order to examine the relationship between TPT and A-966492 or the combination of drugs induced sensitization to 6 MV X-ray, the induction of double-stranded DNA break (DSB) was tested by detecting γ-H2AX expression in U87MG spheroids using Anti-gamma H2A.X phospho S139 antibody (Abcam, UK).

Cells fixed immediately after drug treatments and irradiation. The level of γ-H2AX expressions, shown in figure 2, were calculated by image J software was considered as DSB. Figure 3 shows that in the cells treated with 6 MV X-ray (A-966492 -, TPT- , IR+), the γ-H2AX percentage was significantly higher than the control cells (A-966492 -, TPT- , IR-). Figure 3A,B shows that in the cells treated with A-966492 (A-966492 +, TPT- , IR-), the γ-H2AX percentage was significantly higher than the control cells (A-966492 -, TPT- , IR-), on the other hand the difference between γ-H2AX expression in cells treated with TPT (A-966492 -, TPT +, IR -) was not significant in comparison with control cells ($p > 0.05$), indicating that A-966492 can induce DSB specifically. There was a significant difference in the amount of γ-H2AX positive cells which were treated by A-966492 and 6 MV X-ray (A-

966492 +, TPT- , IR+) or TPT and 6 MV X-ray (A-966492 -, TPT+ , IR+), which confirms that both drugs individually have the capability to enhance radiosensitization at the dose of 1, 1.5 and 2 Gy. According to Figure 3C, the level of γ-H2AX expressions in cells treated with both A-966492 and TPT combined with the dose of 1, 1.5, 2 Gray of 6 MV X-ray was more than cells treated in the group exposed to 6 MV X-ray alone ($P< 0.05$), revealing that combination treatment with A-966492 and TPT enhanced the induction of DSB by radiation.

4. Discussion

Glioblastoma multiform is one of the resistance tumors against radiotherapy. High dose radiotherapy may cause severe side effects. Although adjuvant treatment with Temozolomide was beneficial for some patients but tumor recurrence is inevitable. Radiosensitizer drugs may improve the radiotherapy and much more decrease its side effects. PARP inhibitors and also topoisomerase I inhibitors such as TPT increase sensitivity to radiation [17, 18]. Recently, TPT has been evaluated as an adjuvant treatment for glioma, specifically can be used as a radio-sensitizing agent [19]. A-966492 is a novel and highly potent PARP inhibitor and its ability to cross the blood brain barrier [10] has made it a candidate for the treatment of glioblastoma. However, the effect of A-966492 on malignant glioma has not been reported. Thus, in this study, the radio-sensitizing effect of TPT and A-966492 were evaluated on U87MG spheroids.

In current study 1µM of A-966492 drug as nontoxic dose, has been used for combination treatment with TPT and irradiation with 6 MV X-ray. The concentration of TPT that has been applied for treatments was 1µM. Data obtained from colony counts as an end point on spheroids, showed that TPT in the concentration of 1µM wasn't cytotoxic. Surviving fraction of spheroids that were treated with both radiation and 6 MV x-ray was lower than cells irradiated with different dose of X-ray alone so it confirms that TPT is a potent radiosensitizer drug on U87MG cells. Our results is consistent with Cholpon et al.[11] who showed that Camptothecine (CPT), which is Topoisomerase I inhibitor, has same effect on U87MG cell line. In their study, CPT increased DSBs accompanied by radiation on U87MG cell line. It can be stated that the cytotoxic mechanism of Campothecins is largely S-phase dependent, which is caused by a collision between the replication fork, and that TPT stabilizes the complex which leads to forming DSB [20]. The results of Eyvazzadeh et al.[21] were in accordance with ours as it illustrated that TPT can sensitize U87MG spheroids to radiation by killing cells specifically in the S phase and its topoisomerase I inhibitory effects. Next, we investigated the radio-sensitizing effect of A-966492 and TPT in combination with 6 MV X-ray. Our data showed that, the surviving fraction was similar to that of TPT at the dose of 1Gy and 1.5 Gy; however, by increasing the radiation dose up to 6 Gy the surviving fraction had much more reduction in groups treated with TPT or A-966492. Combination therapy by applying two drugs and irradiation markedly increased radiosensitization. Reduction in the surviving fraction for A-966492 is higher than that for TPT in higher doses ($P < 0.05$), which means that the inhibitory effect of A-966492 is

stronger than TPT. Also, according to Table 1, SER_{10} for A-966492 is significantly higher than SER_{10} for TPT, which confirms that A-966492 is a strong PARP inhibitor and radio-sensitizer. A-966492 enhances radio-sensitivity by inhibiting the repair of SSB and inducing lethal DSB, which leads to radio-sensitization caused by the collapsing of replication forks [18, 22]. As a result, after exposure of U87MG spheroids to A-966492 and irradiation, SSB is converted to DSB during replication [23]. Which confirms the fact that radiosensitization by PARP inhibition is replication dependent [22]. Brazzuol et al. examined radiosensitivity of ABT-888 on GBM cells, and found that the amount of SER_{50} for cells treated with 5μM of ABT-888 and irradiated by 5 Gy X-ray was 1.28 [24]; compared to our data which was 1.39, indicating that 1μM of A-966492 has a higher radio-sensitization on GBM cells. On the other hand, PARP inhibitors have been studied invitro and invivo on different tumoral cell lines, and resulted in enhancing radiosensitivity by factors ranging from 1.3 to more than 2 [25]. This fact is consistent with our current study. The combined effects of TPT, A-966492 and radiation on U87MG spheroids significantly increased the lethal effect of radiation ($p < 0.05$), as it is shown in Table 1, SER_{10} for combined treatment is 1.76. So it can be understood that the cytotoxic effect of A-966492, TPT and radiation together was much greater than that of radiation alone. So radiation therapy accompanied by drugs that improves the treatment outcome by using lower doses of radiation may be applicable. Therefore, patients may benefit from less side effects in the treatment process. Our results are in agreement with Miura et al.'s study. They investigated the combination of Olaparib as PARP inhibitor and CPT as topoisomerase I inhibitor for further radio-sensitization. Since A-966492 increased sensitivity to both radiation and TPT, the combination of A-966492 and TPT had the strongest radiation enhancement ratio amongst other treatment groups.

Shortly after the formation of a double-stranded break by irradiation or cytotoxic drugs, H2AX phosphorylation (γ-H2AX) takes place at the site of DSB and is visible after antibody labeling [26, 27]. Therefore, the proportion of γ-H2AX expression is a known and highly sensitive marker of DSB. Currently, immunofluorescent staining is the method for evaluation of γ-H2AX [28]. Accordingly, we investigated the amount of γ-H2AX expression after the exposure of cells to radiation, A-966492 and TPT. TPT alone did not increase γ-H2AX expression markedly, while A-966492 and radiation did. Radiation combined with A-966492 and TPT expressed the highest γ-H2AX among all of the treatment groups. These findings agreed with the results of the clonogenic assay, and confirmed that the radio-sensitization of A-966492 and TPT are due to the induction of DSB as a result of the inhibition of the SSB repair. Because U87MG cells are considered as highly replicating cell lines, converting single stranded breaks to double stranded breaks in the replication phase of the cell cycle increased lethal damage and led tumor cells to death in the HR repair pathway. Further, our results confirms preclinical studies that had been suggested that the combination of Topotecan with anticancer drugs may suppress tumors activity more effective [29, 30]. Therefore, our observation convincing the evidence that, combination therapy with PARP inhibitors and topoisomerase I

inhibitors may improve radiotherapy treatments of GBM tumors. As A-966492 can cross the blood-brain barrier, it can be a potential therapeutic approach for effective treatment of glioblastoma patients.

Conflict of interest and funding

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Figures and table legends

Figure 1. Cell survival curves of radiation combined with A-966492 and/or TPT. U87MG spheroids were treated with 1 µM A-966492 and/or TPT and irradiated with 6 MV X-ray. Surviving fraction versus dose (Gy). U87MG spheroids treated with (A) 6 MV X-ray and TPT + 6MV X-ray (B) 6 MV X-ray and A-966492 + 6MV X-ray (C) combination of TPT, A-966492 and 6 MV X-ray (D) comparison of all mentioned groups (A, B, C). Values shown are the mean ± SD of three independent experiments.

Figure 2. **γ-H2AX expression using Immunofloresence staining**. Cells were treated with 1 µM of A-966492 and/or 1 µM of TPT, then irradiated with 6 MV X-ray. Cells immediately fixed with paraformaldehyde. Row 1 are cells nuclei, stained with PI and row 2 γ-H2AX expression of cells.

Figure 3. γ-H2AX expression analysed by image J software. Cells were treated with 1 µM of A-966492 and/or 1 µM of TPT, then irradiated with 6 MV X-ray. DNA damage (DSB) was determined by immunofluorescence staining of phosphorylated histone H2AX expression. γ-H2AX plotted versus the dose (Gy). U87MG spheroids treated with (A) 6 MV X-ray and TPT + 6MV X-ray (B) 6 MV X-ray and A-966492 + 6MV X-ray (C) combination of TPT, A-966492 and 6 MV X-ray (D) comparison of all mentioned groups (A, B, C). Symbols represent mean ± SD of at least three independent experiments.

Table1. Radiobiological parameter values. Mean values of α, β and SER_{50} of U87MG cells, estimated by fitting the cell survival to the LQ model.

Treatment	α±SD	β±SD	SER_{50}	SER_{10}
X-ray	0.00±0.006	0.04±0.002	-	-
X-ray + TPT	0.00±0.021	0.06±0.007	1.16	1.16
X-ray + A-966492	0.02±0.025	0.07±0.010	1.39	1.39
X-ray + A-966492 + TPT	0.04±0.061	0.09±0.028	1.53	1.76

Table1. Radiobiological parameter values. Mean values of α, β and SER_{50} of U87MG cells, estimated by fitting the cell survival to the LQ model.

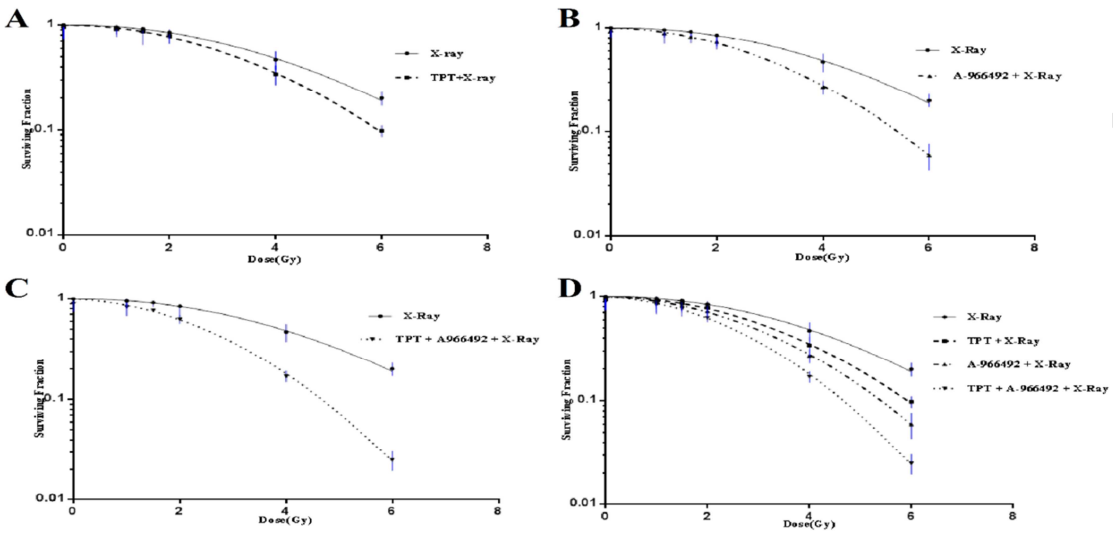

Figure 1. Cell survival curves of radiation combined with A-966492 and/or TPT. U87MG spheroids were treated with 1 µM A-966492 and/or TPT and irradiated with 6 MV X-ray. Surviving fraction versus dose (Gy). U87MG spheroids treated with (A) 6 MV X-ray and TPT + 6MV X-ray (B) 6 MV X-ray and A-966492 + 6MV X-ray (C) combination of TPT, A-966492 and 6 MV X-ray (D) comparison of all mentioned groups (A, B, C). Values shown are the mean ± SD of three independent experiments.

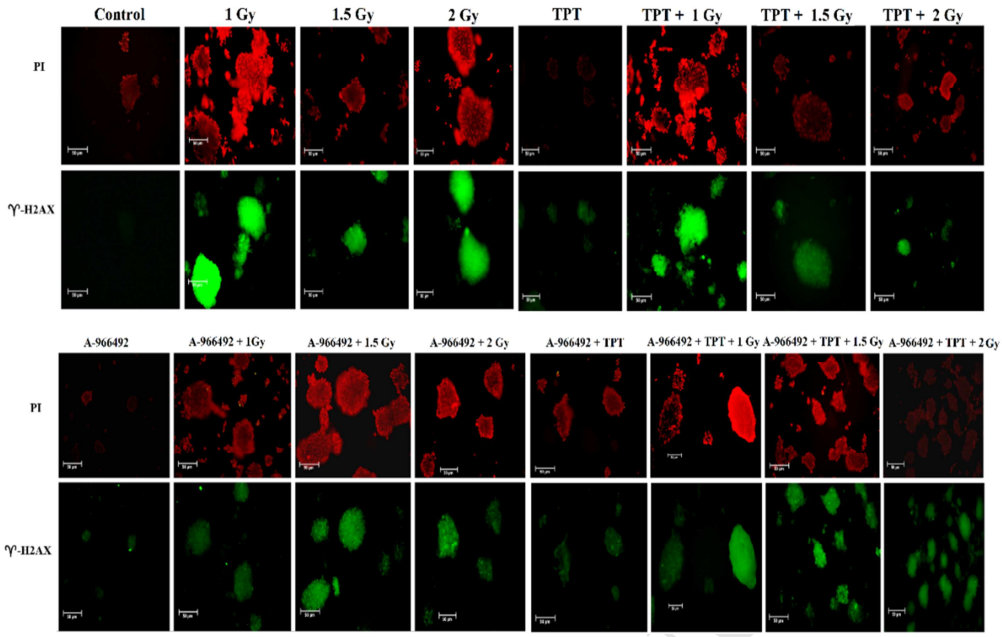

Figure 2. **γ-H2AX expression using Immunofloresence staining**. Cells were treated with 1 µM of A-966492 and/or 1 µM of TPT, then irradiated with 6 MV X-ray. Cells immediately fixed with paraformaldehyde. Row 1 shows cells nuclei, stained with PI and row 2 shows γ-H2AX expression of cells.

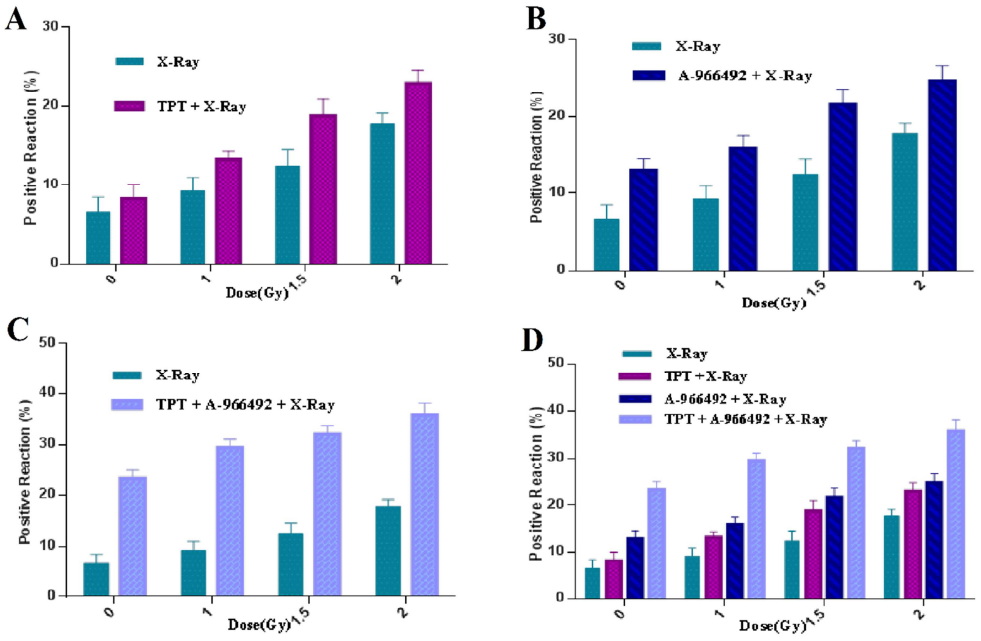

Figure 3. γ-H2AX expression analysed by image J software. Cells were treated with 1 µM of A-966492 and/or 1 µM of TPT, then irradiated with 6 MV X-ray. DNA damage (DSB) was determined by immunofluorescence staining of phosphorylated histone H2AX expression. γ-H2AX plotted versus the dose (Gy). U87MG spheroids treated with (A) 6 MV X-ray and TPT + 6MV X-ray (B) 6 MV X-ray and A-966492 + 6MV X-ray (C) combination of TPT, A-966492 and 6 MV X-ray (D) comparison of all mentioned groups (A, B, C). Symbols represent mean ± SD of at least three independent experiments.

Highlights

- A-966492 is an effective drug with significant radiosensitivity on U87MG spheroids.
- Combination of A-966492 and TPT had synergistic radiosensitizing effect.
- Applying PARP and topoisomerase I inhibitors can improve radiotherapy treatment.