Effect of metformin/irinotecan-loaded poly-lactic-co-glycolic acid nanoparticles on glioblastoma: *in vitro* and *in vivo* studies

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**Aim:** The present study was designed to evaluate the effects of irinotecan hydrochloride (IRI)- or metformin hydrochloride (MET)-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles (NPs) for the treatment of glioblastoma multiforme using *in vitro* neuron and U-87 MG glioblastoma cell cultures and *in vivo* animal model. **Methods:** The cytotoxic and neurotoxic effects of pure drugs, blank NPs and MET- and IRI-loaded PLGA NPs were investigated *in vitro* (using methylthiazolyldiphenyl-tetrazolium bromide assay) and *in vivo* (using Cavalieri’s principle for estimation of cancer volume). **Results:** 1 and 2 mM doses of MET and MET-loaded PLGA NPs, respectively, significantly reduced the volume of extracted cancer. **Conclusion:** Consequently, MET- and IRI-loaded PLGA NPs may be a promising approach for the treatment of glioblastoma multiforme.

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**Keywords:** brain tumor • Cavalieri’s principle • irinotecan • metformin • nanoparticles • PLGA

Glioblastoma multiforme (GBM) is the most common and lethal primary brain tumor in adults; originating from glial cells [1–3]. This solid tumor is responsible for 77% of all primary brain tumor-related mortality [4]. The localization of the tumor, the overall health and the age determines what kind of therapy the patients need to receive. GBM treatment requires a multidisciplinary approach, including surgery, radiotherapy and chemotherapy. Despite the advances in all treatments, malignant GBM therapy remains a great challenge [5,6]. Current chemotherapeutics are limited because of the low selectivity to cancer cells, which causes unpleasant side effects and harm the healthy cells [6]. For effective treatment of malignant central nervous system cancers, the GBM cells must be killed selectively or with local application of drug delivery systems [7]. Therefore, it is crucial to understand the development of technology based on drug delivery systems, especially nanoparticles (NPs) to treat GBM.
DNA topoisomerase I (Top 1) is a primary enzyme, which construct the structural changes in DNA topology with catalyzing the concerted breaking and reuniting of DNA strands during natural cellular growth [8,9]. Overexpression of Top 1 was linked to several cancers, including lung, colorectal and breast cancers [10]. Irinotecan hydrochloride (IRI) is a semisynthetic competitive analog topoisomerase-I inhibitor, which inhibits nuclear DNA topoisomerase I, leading to single-strand DNA breaks, in turn inducing apoptosis [8,9]. Thence, IRI could be an effective chemotherapeutic agent, showing a high activity against a wide spectrum of malignancies, including GBM [8,9,11]. IRI-loaded liposome has demonstrated considerable therapeutic activity against colorectal cancer [10].

Metformin hydrochloride (MET), an antihyperglycemic agent, is frequently used for treatment of Type 2 diabetes. Recently, it has been proposed that MET could have a protective effect against neoplastic diseases. The direct anticancer effects are associated with independent and dependent AMPK activation that lead to inhibition of mTORC1 [12]. Furthermore, MET has a variety of mechanisms by which cancer growth and carcinogenesis can be retarded [13,14].

Due to their excellent pharmacokinetic properties, polymeric NPs have been used as anticancer drug delivery [15,16]. Poly-lactic-co-glycolic acid (PLGA), approved by the US FDA, has a predictable biodegradation behaviors and improved mechanical properties. It shows a good biocompatibility with cells and tissues [17]. In this context, the aim of the present study was to investigate the potential effects of MET/IRI-loaded PLGA NPs on in vitro and in vivo GBM models. In this view, PLGA was selected as the polymer for preparation of MET- and IRI-loaded NPs (MET-loaded PLGA NPs and IRI-loaded PLGA NPs). The drug-loaded NPs were prepared and characterized with respect to particle size, zeta potential, morphology, drug loading and MET and IRI in vitro release behavior from the NPs. In vitro MTT test was employed to quantify the cell proliferation on neuron cells and U-87 MG cell line. Additionally, the antitumor effects of pure drug as well as the NPs and tumor volumes estimated by Cavalieri’s principle were evaluated in pathologically prepared tumor regions slice in in vivo animal model.

**Methods**

**Chemicals & reagents**

IRI (CAS Number: 136572-09-3) was purchased from LC Laboratories (MA, USA). PLGA (Resomer® RG 502, MW 7000–17,000 Da), Dulbecco’s modified Eagle medium, fetal calf serum, neurobasal medium (NBM), Roswell Park Memorial Institute 1640, phosphate-buffered saline (PBS), antibiotic antimycotic solution (100×), L-glutamine and trypsin-EDTA were obtained from Sigma-Aldrich (MO, USA). Metformin hydrochloride was a generous gift from Sandoz (Novartis, Istanbul, Turkey).

**Preparation of MET-PLGA NPs & IRI-PLGA NPs**

For the preparation of MET-loaded PLGA NPs, a 150 mg PLGA was dissolved in 10 ml ethyl acetate before dropping into 20 ml of polyvinyl alcohol aqueous solution (3%, w/v) containing MET. The polymer solution was emulsified using an ultrasonic probe (with 60% power; Sonoplus HD 2070; Bandelin Electronics, Berlin, Germany) for 15 min. Afterward, the organic phase was removed using rotary evaporator at 45°C. Following centrifugation at 5000 r.p.m. for 5 min (15°C), the collected supernatant was centrifuged at 13,500 r.p.m. for 50 min (15°C) to obtain NPs. The NPs were resuspended in ultrapure water, lyophilized for 24 h (-55°C; 0.021 mbar) and stored at -20°C in closed and sealed containers.

For IRI-loaded PLGA NPs, a 50 mg of PLGA was dissolved in dichloromethane and then, IRI solution in acetonitrile was added drop by drop into PLGA solution to obtain the organic phase. Subsequently, the organic phase was dropped into 20 ml polyvinyl alcohol aqueous solution (3% w/v) and emulsified using an ultrasonic probe (with 60% power) (Sonoplus HD 2070; Bandelin Electronics, Berlin, Germany) for 9 min. Afterward, the rest of the procedures were the same as aforementioned in MET-PLGA-loaded NPS. Blank NPs were prepared using same procedures mentioned above without adding IRI and MET.

**Characterization of MET-PLGA NPs & IRI-PLGA NPs**

The surface morphology of the NP formulations was visualized utilizing scanning electron microscope (SEM; NOVA NanoSEM 430, FEI, Brno, Czech Republic). The SEM images of lyophilized NPs mounted on metal stubs and scattered with gold were captured. The mean particle sizes and zeta potentials of the NPs were determined by Zetasizer 3000 HS (Malvern Panalytical Ltd, Malvern, UK). The MET and IRI contents in NPs were determined using a validated UV method (UV–Vis spectrophotometer; Beckman Coulter-DU® 730, CA, USA). The drug
Drug-loaded NPs are more effective on glioblastoma

Research Article

Contents were measured at 232 and 221 nm for MET and IRI, respectively, and then the encapsulation efficiency (EE%) values of the MET- and IRI-PLGA NPs were calculated.

In vitro drug release from matrix was estimated using a dialysis sac (molecular weight cut-off 14,000 Da) immersed in PBS pH 7.4 at 37 ± 0.5°C and 50 r.p.m. At different time intervals, the samples (drug-loaded PLGA) were withdrawn from the medium and replaced with the same volume of fresh PBS. The samples (2 ml) were centrifuged at 12,500 r.p.m. for 10 min and the drug contents in the supernatants were measured as stated above.

In vitro studies

Cell cultures

Neuron cell cultures were prepared using nine new-born Sprague–Dawley rat fetuses as described elsewhere[18–20]. The cells were seeded into 48-well culture plate at density of 1 × 10⁵ cells. Then the cells were treated with different concentrations of IRI (1, 10 and 100 μM) [21], MET (0.25, 0.5, 1 and 2 mM) [22], blank PLGA NPs, IRI-loaded PLGA NPs and MET-loaded PLGA NPs and incubated for 48 h (5% CO₂; 37°C). As a control, a 150 μl NBM (Gibco Sigma, MO, USA) was only added to each well for 48 h. Then, MTT assay was conducted using commercially available kit (Cayman Chemical, MI, USA). Briefly, MTT reagent (10 μl) was added to each well and the plates were incubated (5% CO₂; 37°C) for 4 h. Thereafter, the medium was discarded and A 100 μl of dimethyl sulfoxide was added to each well. The optical density was determined at 570 nm using Multiskan™ GO Microplate Spectrophotometer reader (Thermo Fisher Scientific Oy, Ratastie, Finland) and the cell viability (%) was calculated [19].

U-87 MG glioblastoma cells were harvested with 0.25% trypsin–EDTA and suspended with Roswell Park Memorial Institute 1640 medium containing 15% FBS, 1% L-glutamine and 1% penicillin–streptomycin. Cells were seeded in 25 cm² flasks. After reaching the proper volume, they were seeded in 96-well plates. Thus, each well contained a 100 ml medium with 1 × 10⁵ cells. As a control, only 150 μl Dulbecco’s modified Eagle medium (Gibco Sigma) was added to each well, then drug application procedure and MTT assay was repeated as described above [2].

In vivo studies

A rat model for GBM

U-87 MG cells were used to develop the experimental rat brain tumor model. Male Sprague–Dawley rats (n = 52) weighting 210 ± 10 g were randomly divided into 13 groups (n = 4/group). Before experimental protocol, the animals orally received 35 mg cyclosporine (immunosuppressant drug) for two times (0 and 24 h) [23]. The rats anesthetized with sevoflurane were placed on the stereotactic device. The skull was sterilized with 10% povidone–iodine. Using a scalpel and fine-tipped scissor, the scalp was opened up from neck to the middle of eye and parted with forceps. Cells (1 × 10⁶) in NBM were slowly injected intracranially (stereotactically) using Hamilton microsyringe into the brain at a depth of 3.8 mm (z) into the right striatum (coordinates with regard to Bergman: -1 mm [x] and 2.5 mm [y]) nigrostriatal region[23]. On day 7, two rats were randomly chosen and the histopathologic changes were investigated to prove the presence of cancer.

Drug administration

On day 15, IRI (100 and 1000 μM) [21] and MET (30 and 300 mM) [24], blank PLGA NPs and IRI-PLGA-loaded NPs (equivalent to 100 and 1000 μM of IRI), MET-PLGA-loaded NPs (equivalent to 30 and 300 mM of MET); IRI-PLGA-loaded NPs and MET-PLGA-loaded NPs combinations (IRI-PLGA-loaded NPS + MET-PLGA-loaded NPs) at different concentrations (equivalent to 1000 μM of IRI and 300 mM of MET; and also equivalent to 100 μM of IRI and 30 mM of MET) were injected directly into (cancer region) the brain [25]. For control group, only U-87 MG cells were implanted into brain. After 48 h, animals were decapitated under sevoflurane anesthesia.

Histopathological determination

Removed brains were fixed for 48 h in 10% buffered formaldehyde. The processed right hemisphere dehydrated through and processed by graded alcohols and xylene. Then, immersed in paraffin series and embedded in fresh paraffin. Five micrometer sections were obtained via Leica (2450, Turkey) microtome device. Among each ten obtained section, the first one was taken but the other nine were thrown, respectively. Totally 29 sections were chosen and stained with hematoxylin and eosin [24]. For the estimation of tumor volume, Cavalieri’s principle
Table 1. Histopathological finding of glioblastoma multiforme cancer.

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<th>Experiment</th>
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(A) Hematoxylin and eosin (HE) stain, ×4200 μm; (B) HE stain, ×10,100 μm; (C) HE stain, ×2050 μm. Black arrow: Necrotic tissue; Black arrow head: Necrotic tissue and hemorrhage; Blue arrow: Only hemorrhage; White star: Glioma tissue; Black spring: Healthy tissue.

IRI: Irinotecan hydrochloride; MET: Metformin hydrochloride; NP: Nanoparticle; PLGA: Poly-lactic-co-glycolic acid.
Table 1. Histopathological finding of glioblastoma multiforme cancer (cont.).

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IRI: Irinotecan hydrochloride; MET: Metformin hydrochloride; NP: Nanoparticle; PLGA: Poly-lactic-co-glycolic acid.
(Stereo Investigator® version 8.0, Micro Bright Field, VT, USA) was used for volumetric measurements of selected tumor regions slice (totally 29 different slice).

Statistical analysis
The statistical analysis was performed using one-way analysis of variance and Tukey’s honest significant difference test (SPSS 20.0 Software for Windows). A p-value <0.05 was considered as statistically significant.

Results
Figure 1 shows the SEM images of both MET- and IRI-PLGA NPs. The images revealed that the NPs were approximately spherical and in the nanosize range.

The mean particle sizes and zeta potential values were 300 ± 5.87 and 216 ± 4.48 nm and -0.121 ± 0.26 and -16.37 ± 1.86 mV, for MET- and IRI-PLGA NPs, respectively. Thence, the MET- and IRI-PLGA NPs were stored in lyophilized powder. The encapsulation efficiency values for MET- and IRI-PLGA NPs were low (2.30 ± 0.41% and 12.39 ± 0.66%, respectively), due to the leakage of the drugs to the external medium during preparation of NPs. Approximately, 40 and 93% of MET was released from MET-PLGA NPs and approximately 43 and 100% of IRI was released from the IRI-PLGA NPs within 1 and 48 h, respectively, in PBS (pH 7.4) (Figure 2).

In cell culture studies, the effects of MET (0.25, 0.5, 1 and 2 mM), blank PLGA NPs and MET-loaded PLGA NPs on neuron and U-87 MG cells are shown in Figure 3. The MTT assay confirmed that blank PLGA NPs did not exert any potential cytotoxic effects on neuron and U-87 MG cells compared with control sample (Figures 3 & Figure 4). However, the high MET concentrations (1 and 2 mM) and MET-loaded PLGA NPs significantly decreased cell viability on both cell lines (p < 0.001, p < 0.05; Figure 3) compared with control. It has to be noted that there were no significant differences between the control groups (negative control and blank PLGA NPs and pure MET) and MET-loaded PLGA NPs (at low doses) on both neuron and cancer cells (p > 0.05; Figure 3). On the other hand, the cell viability ratio of IRI (1, 10 and 100 μM), blank PLGA NPs and IRI-loaded PLGA NPs on neuron and U-87 MG cells were demonstrated in Figure 4. Notably, 100 μM IRI and IRI-loaded PLGA NPs significantly decreased cell viability compared with the control (p < 0.001). Additionally, 10 μM IRI and IRI-loaded PLGA NPs significantly decreased U-87 MG cells viability (p < 0.05; Figure 4). Finally, 1 μM concentration IRI and IRI-loaded PLGA NPs did not show any significant effect on both cell lines compared with control group (Figure 4).

In pathological examination, U-87 MG cells (implanted brain region of cancer control) showed a significant reduction in the mitotic index and other histological characteristics (hemorrhagic and inflammation area) that indicate a less invasive/proliferative tumor (Table 1).

The volume of cancer was estimated by Cavalieri’s principle and the results are shown in Figure 5. We have obtained a significant reduction in the volume of cancer for all pure drugs and drug-loaded NPs groups compared with cancer control group (p < 0.001). There was no significant difference in the tumor volumes between cancer control group and blank PLGA NPs group (p > 0.05). In terms of tumor volume reduction rates, IRI-loaded PLGA NPs and MET-loaded PLGA NPs combinations (IRI-loaded PLGA NPs + MET-loaded PLGA NPs 1000 μM–300 mM; tumor volume: about 17%) and also IRI-loaded PLGA NPs (equivalent to 1000 μM; tumor volume: about 12%) were found to be more effective (p < 0.001) compared with other groups (pure drugs and the other drug-loaded NPs groups). The in vivo study showed that all drug-loaded NPs induced a significant reduction in tumor volume compared with the corresponding pure drugs (p < 0.05; Figure 5).

Discussion
The cell and tissue distribution profiles of anticancer agents can be controlled by the use of anticancer drug-loaded NPs; the approach that can decreases the systemic side effects and increases the antitumor efficacy [26]. Herein, we investigated the effects of MET- and IRI-loaded PLGA NPs both in in vivo and in vitro glioma models. For this purpose, first, MET- and IRI-loaded PLGA NPs were prepared and in vitro characterized. MTT assay was used to evaluate the cell viability of pure drugs (MET and IRI), blank PLGA NPs and MET- and IRI-loaded PLGA NPs on neuron and U-87 MG cells. To evaluate the in vivo antitumor efficacy of drug-loaded NPs, pathological studies were performed as well and tumor volumes were estimated using Cavalieri’s principle method.

MET is a cytostatic agent which activates AMPK, leading to inhibition of mTOR-signaling pathway [27,28]. Owing to this mechanism, Evans et al. [13] proposed that MET might reduce the risk of tumor and overall cancer-related deaths among patients with Type 2 diabetes. Previous reports have indicated that MET showed
Drug-loaded NPs are more effective on glioblastoma

Figure 1. Scanning electron microscope images of MET-PLGA nanoparticles (A) and IRI-PLGA nanoparticles (B). IRI: Irinotecan hydrochloride; MET: Metformin hydrochloride; PLGA: Poly-lactic-co-glycolic acid.

anticancer activity on solid tumors, such as endometrial carcinoma [29], medullary thyroid cancer [30], pancreatic cancer [31], gastric cancer [32] and breast cancer [33]. Recently, it was suggested that AMPK-targeting drugs, such as MET with enhanced blood–brain barrier permeability could be evolved as a potential agent for treatment of GBM [34,35]. In the same line, Sesen et al. [36] indicated that MET can induce GBM cell death, autophagic and apoptotic processes in vitro and in vivo that was potentially due to AMPK and Redd1 activation, mitochondrial energetic and mTOR pathway inhibition. At the same time, MET was able to inhibit migration of U-87 MG cells,
because of invasive behavior of GBM mainly uncontrolled cellular proliferation [27,28]. On the other hand, IRI is an important drug for cancer therapy, especially, in combination with other chemotherapeutic agents [37]. IRI, first, was used for treatment of small and non-small-cell lung, ovarian and cervical cancer in Japan [38]. Two years later, it was first approved for treatment of metastatic colorectal cancer in the USA [39]. IRI has shown a significant anticancer activity against an extensive panel of subcutaneous and intracranial human GBM, ependymoma and medulloblastoma xenografts [40,41] in animal studies. Likewise, a synergistic effect was demonstrated when brain tumor xenografts was treated with alkylating agents, including carmustine and temozolomide followed by IRI [40,41]. Friedman et al. [42] reported that IRI has a favorable activity in adults’ patients with recurrent malignant glioma. Notwithstanding, other studies have demonstrated the anticancer activity of IRI and MET, yet it has not been explored with IRI- and MET-loaded NPs in GBM models. In this study, MET- and IRI-loaded NPs have shown a burst release effect within the first 2 h and later they showed a slow released effect owing to adsorption of the drugs on the surface of NPs (Figure 2). In vitro cytotoxic effects of pure drugs and NP formulations were determined in neuron and U-87 MG cells via MTT assay. The high concentrations of MET (1 and 2 mM) and MET-loaded PLGA NPs exhibited significant cytotoxicity on both cells. Additionally, it was found that the cytotoxic effects of PLGA NPs and pure MET on neuron and cancer cells were similar. Low concentrations (0.5 and 0.25 mM) of MET and MET-loaded PLGA NPs showed lower cytotoxic effects in both cells. Moreover, the high concentrations (100 μM) of IRI and IRI-loaded PLGA NPs have shown significant cytotoxic effects on cancer and neuron cells. Ten micromolar of IRI and IRI-loaded PLGA NPs showed cytotoxic effect on U-87 MG cells. In the presence
Drug-loaded NPs are more effective on glioblastoma

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In vitro cytotoxicity effects of MET

**Figure 3.** In vitro viability ratio of metformin hydrochloride (pure drug: 0.25–2 mM), blank poly-lactic-co-glycolic acid nanoparticles and metformin hydrochloride-poly-lactic-co-glycolic acid nanoparticles (equivalent to 0.25–2 mM metformin hydrochloride) on neuron and U-87 MG cells (n = 6/group). Significant differences at *p < 0.05 and **p < 0.001 compared with control group.

MET: Metformin hydrochloride; NP: Nanoparticle; PLGA: Poly-lactic-co-glycolic acid.

In vitro cytotoxicity effects of IRI

**Figure 4.** In vitro cytotoxic effects of irinotecan hydrochloride (pure drug: 1–100 μM), blank poly-lactic-co-glycolic acid nanoparticles and irinotecan hydrochloride-poly-lactic-co-glycolic acid nanoparticles (equivalent to 1–100 μM of irinotecan hydrochloride) on neuron and U-87 MG cells (n = 6/group). Significant differences at *p < 0.05 and **p < 0.001 compared with control group.

IRI: Irinotecan hydrochloride; NP: Nanoparticle; PLGA: Poly-lactic-co-glycolic acid.

of PLGA NPs, the neurotoxic effects of IRI on neuron cells were lower than that of pure IRI compared with the cytotoxic effects of them on cancer cells, with no significant difference (p > 0.05; Figure 4).

In this study, we also aimed to evaluate the effects of pure drugs (MET and IRI) and drug-loaded PLGA NPs on GBM size by Cavalieri's principle (Figure 5). Obviously, there were no significant differences in in vivo cancer spread rate between blank PLGA NPs and control group (p > 0.05). All drug-loaded NPs caused a significant reduction in
tumor volume (volume of tumors: about 12–38% for IRI- or MET-loaded NPs and NP combinations) compared with corresponding pure drugs (volume of tumors: approximately 45–68% for pure IRI and MET), blank NPs (tumor volume: approximately 98%) and cancer control (tumor volume: 100%) (p < 0.05; Figure 5). NPs have many advantages, such as protecting the drugs from degradation, sustainable releases of the drug, increasing the drug retention times in tumor and increasing drug circulation and payloads [43–45].

In conclusion, NP-based technology was utilized to achieve better therapeutic efficiency. The current study showed that MET- and IRI-loaded PLGA NPs may be a promising approach to treat GBM.

**Summary points**

- Metformin hydrochloride (MET)- and irinotecan hydrochloride (IRI)-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles (NPs) were prepared and characterized.
- Morphology, size and zeta potential and drugs uptake release potential were evaluated.
- *In vitro* studies reveal that the MET- and IRI-loaded PLGA NPs have the potential to decrease neurons and U-87 MG glioblastoma cells viability.
- *In vivo* studies disclose that the MET- and IRI-loaded PLGA NPs have the capacity to reduce tumor size in Sprague–Dawley rats.

**Financial & competing interests disclosure**

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No writing assistance was utilized in the production of this manuscript.

**Ethical conduct of research**

This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (42190979-01 – 02/2411).
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