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RESEARCH PAPER

The effects Metformin/Irinotecan-loaded PLGA nanoparticles on glutamate re-uptake time and alteration EAAT1 gene expression level in vitro

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ABSTRACT

Objective(s): The present study was designed to evaluate of Metformin/Irinotecan-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles (NPs) effects on glutamate re-uptake time and receptor expression status in both glioblastoma multiforme (GBM) and cortex neuron cultures. The study was performed on glioblastoma cell line and primer cortex neuron.

Materials and Methods: The re-uptake time and gene expression status of pure drugs and MET- or IRI-loaded-PLGA NPs on healthy neuron cells and U-87 MG cell line were investigated by using glutamate specific voltammetry electrodes technique and real time PCR.

Results: Both MET and MET-PLGA NPs (1 and 2 mM) exhibited significant cytotoxicity on both U87MG and neuron cells. MET and MET-PLGA NPs (0.5 mM) showed lower cytotoxic effects on both cells. IRI and IRI-PLGA NPs (100 µM) had significant cytotoxic effects on both cell lines.

Conclusion: All drug-loaded NPs caused a significant reduction in glutamate reuptake time compared with free drugs, blank NPs and cancer cells control groups. Consequently, MET- and IRI-loaded PLGA NPs may be a promising approach to treat GBM.

Keywords: *EAAT1, Irinotecan, Metformin, PLGA, Voltammetry*

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INTRODUCTION

Glioblastoma multiforme (GBM) is most aggressive of all (malignant) primary brain tumor, with a median survival around 12–14 months [1]. Despite of all surgical, chemo and radiotherapy advances, malignant GBM therapy remains a great challenge [2]. GBM increases glutamate level in synaptic area inducing toxicity to neuron cells [3]. Glutamate have important role in cell-cell communication as excitatory neurotransmitter. Glutamate potentially has ability to induce neurotoxicity and degeneration when present out of physiological condition transmiting all electrical current from presynaptic neuron to post synaptic neuron (synaptic area) [4-6]. Current chemotherapeutics are limited by low selectivity toward cancer cells, which causes unpleasant side effects and harmful to healthy cells. Since the neurons are not able to be regenerated, the GBM cells must be killed selectively. Hence, it is crucial to develope a new technology-based, and efficient

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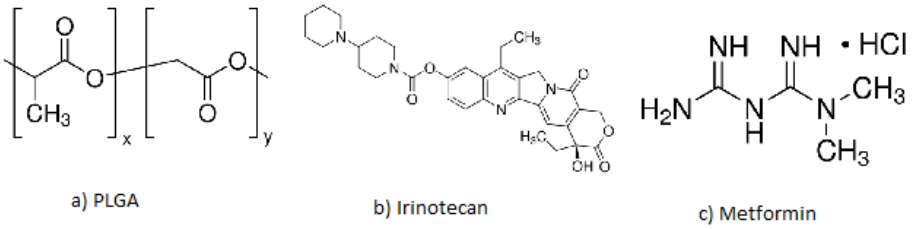

Fig 1. Chemicals structure of poly-D,L-lactide-co-glycolide, irinotecan and metformin

tumor cell-targeted therapy for treating GBM.

Polymeric nanoparticles (PNPs), especially those prepared by poly-(DL-lactic-co-glycolic) acid (PLGA), are widely used as tumor-targeting system to deliver drugs to cancers cells [7]. Metformin hydrochloride (MET) is frequently used to treat type II diabetes, but it has been recently proved that MET can be useful in cancer therapy as well [8, 9]. MET regulates hyperglycemia by activating 5'-adenosine monophosphate-activated protein (AMPK). Previous studies have shown that AMPK has an inhibitory role on cancer cells by down regulating the mammalian target of rapamycin (mTOR) (10). Furthermore, MET is used against gastric, skin, breast and T98G (type of glioblastoma cancer) cancers [11-14].

Irinotecan hydrochloride (IRI), a semi-synthetic competitive analogue of topoisomerase-I inhibitor, is an important and affective chemotherapeutic. It shows high activity against a wide spectrum of malignancies, including GBM [15]. It was reported that irinotecan is useful for colorectal, breast, and glioblastoma cancers [16-19].

Voltammetry is a technique for measuring the concentration of wide range of compounds through their oxidation at an inert electrode surface. Previously it has been proved that this technique can be effectively used for measuring the release and re-uptake amounts of neurotransmitters [20]. Unlike routine methods, voltammetric methods rapidly measure the dynamic properties of neurotransmitters. Because of these features, voltammetry has been shown to cover all limitations of microdialysis method [21].

In this study, we investigated the effect of metformin/irinotecan PLGA nanoparticleson GBM and neurons. *In vitro* voltammetry test and PCR were employed to quantify the glutamate re-uptake time, receptor and transporter expression levels on rat primary brain cortex neuron cells and GBM cultures.

MATERIALS AND METHODS

Chemicals and reagents

Irinotecan hydrochloride (IRI), poly-D,L-lactide-co-glycolide (PLGA, Fig 1), Dulbecco modified Eagles medium (DMEM), fetal calf serum (FCS), neurobasal medium (NBM), phosphate buffer solution (PBS), antibiotic antimitotic solution (100×), L-glutamine and trypsin–EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Metformin hydrochloride (MET) was a generous gift from Sandoz (a Novartis Company, Turkey).

Preparation of MET-PLGA NPs and IRI-PLGA NPs

NPs were obtained as reported in our previous study [22]. For the preparation of MET-PLGA NPs, PLGA was dissolved in ethyl acetate, then was dropped into 3% PVA aqueous solution containing MET and emulsified with an ultrasonic probe (Sonoplus HD 2070; Bandelin Electronics, Germany).

The organic phase was removed using a rotary evaporator at 45°C. Following centrifugation at 5000 rpm, the collected supernatant was centrifuged at 13500 rpm to collect NPs. The NPs were resuspended in ultrapure water and lyophilized for 24 hr. For IRI-PLGA NPs, PLGA was dissolved in dichloromethane and then, IRI solution in acetonitrile was added dropwise into PLGA solution to obtain the organic phase. The organic phase was added into PVA aqueous solution (3%, w/v) and emulsified using an ultrasonic probe. The rest of the procedures were similar to aforementioned method used for the preparation of MET-PLGA NPs. Blank NPs were prepared using the same procedures mentioned above without adding MET or IRI.

Characterization of MET-PLGA NPs and IRI-PLGA NPs

The surface morphology of the nanoparticle

formulations was examined by using scanning electron microscope (NOVA NanoSEM 430, FEI, Czech Republic). The mean particle sizes and zeta potentials of the NPs were determined by the Zetasizer 3000 HS (Malvern Instruments, UK). The MET and IRI contents iof NPs were determined using a validated UV method (UV-Vis spectrophotometer; Beckman Coulter- DU® 730, USA) and measurements were performed at 232 nm for MET and 221 nm for IRI. Then the encapsulation efficiency (EE%) values of the MET- and IRI-PLGA NPs were calculated.

Cell culture

Neuron culture

Primary neuron cortex culture was isolated and cultured as previously described [22]. Briefly cortical neurons were extracted from the brain of one-day-old Sprague Dawley rats. The cells were centrifuged at 1000 *g* for 5 min and the cells were suspended in neuron basal medium (NBM) supplemented with 10% fetal bovine serum, 2% B27 supplement (50X) (Life Technologies, Carlsbad USA) and 0.1% penicillin/streptomycin at a density of 1×10^5 cells. Each well contain 100 µl medium placed in a humidified incubator (37 ˚C with 5% CO_2)(23, 24).

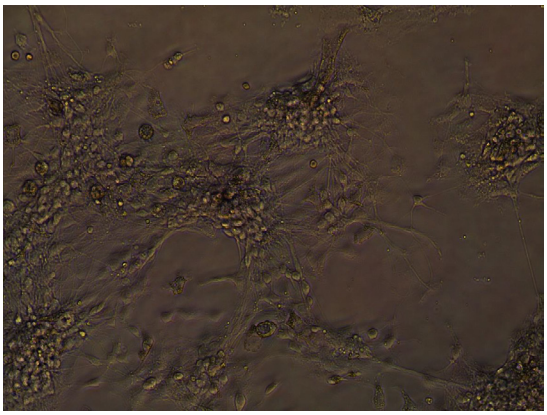

Fig 2. Neuron cells culture view (20× magnitude)

Glioblastoma cell line

U87MG cell line is used widely for modeling of aggressive brain cancer. The U87MG cells were obtained from Ataturk University, Department of Medical Pharmacology, Faculty of Medicine (Erzurum, Turkey). Prior to experiments, cells were grown in 25 cm^2 culture flasks in RPMI 1640 (Sigma-Aldrich) supplemented with 1% glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum at 37 ˚C in a humidified (95%) incubator with CO_2 (5%). The cultured cells were trypsinised with 0.25% trypsin/EDTA for 3 min and then were seeded in a 96-well plate, each well received 100 µl medium with 1×10^5 cells (2).

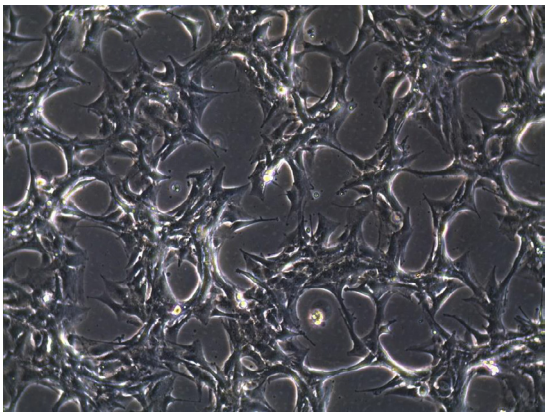

Fig 3. U87MG cells culture view (20× magnitude)

Drug treatment

Cells were treated with each drug separatly in 12 separate groups. Either free or encapsulated IRI (1 and 10 µg) and MET (0.5, 1 and 2 mM) in PLGA and control groups were added to cells (normal control, cancer control and PLGA control).

Voltammetry

Microelectrodes and calibration

Microelectrodes were obtained from Pronexus Analytical (Stockholm, Sweden). The real-time monitoring of rapid changes in extracellular levels of glutamate in the central nervous system was provided by fast analytical sensing technology (FAST). S2 type, glutamate oxidase and nafion-coated multisite ceramic microelectrodes was used in this study. The microelectrodes have platinum (Pt) recording sites with Pt connecting lines. Calibration tests were performed amperometrically in 0.7 voltage for *in vitro* voltammetry. The ceramic microelectrode amplifies head stage by being attached to a FAST 16 system. An Ag/AgCl commercial electrode was also attached to the head stage which functioned as the reference electrode. Calibration tests were involved placement of the electrodes in a stirred 40-ml of 0.1 M phosphate buffered saline (PBS; pH 7.4 Sigma, St. Louis, MO, USA) solution. Different layers on microelectrode were shown in Fig 4 [21].

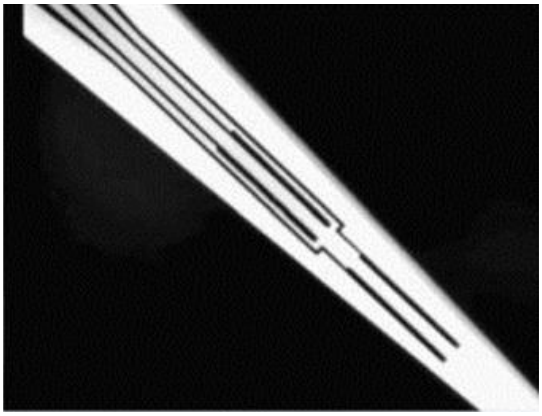

Fig 4. The S2 electrode view of FAST 15 device. A) is self refrence and B) is main sensore surface (contained GluOx/ FAD enzyme nafion)

L-glutamate+H_2O+GluOx/FAD → α-ketoglutarate + NH_3 + GluOx/$FADH_2$GluOx/$FADH_2$ + O_2 → GluOx/ FAD + H_2O_2

In vitro voltammetry

Microelectrodes were used to record the re-uptake parameters of glutamate from the cell culture wells. After calibration, culture plates were placed in the middle of a circulating water bath that holds the temperature constant at 37 °C. Electrodes were carefully placed in wells. For determmining the L-glutamate re-uptake, a solution of L-glutamate (10^{-5} M) was injected into the culture media, followed by the determination of the 80% of reuptake time (t_{80}) [25, 26].

PCR

RNA isolation

Isolation was performed by using the Qiagen RNA isolation kit. Briefly, 1 µg/ml Qiazole solution was added to cells for 5 minutes. Then, 200 µl chloroform was added in 15 seconds, and kept at room temperature for 2-3 minutes. Both samples and colorless fluid of the top were transferred to another tube and vortexed by adding ethanol. A sample (700 µl) was taken and centrifuged at room temperature at 8.000 g for 15 seconds [27].

cDNA synthesis

2 µl from the genomic DNA wipeout buffer solution and 1 µg RNase free water were prepared to have a total volume of 14 µl. The mixture were kept at 42 degrees for 2 min, then they were placed in ice. Reverse transcription master mix (1 µl), Quant script RT buffer (5x4 µl), RT primer mix (1 µl) and RNA (14 µg) were mixed and placed in the RT-PCR device. This mixture was heated at 42 degrees for 15 min and subsequently at 95 degrees for 3 min and then it was kept at -20 degree.

Determination of EAAT1 expression

Right and left primary (Realtime ready, Roche, Switzerland) 0.25 µl, probe 0.15 µl, cDNA 3 µl, master mix 3 µl and 12.75 distilled water were added in each tube and adjusted to have a final volume of 20 µl. It was incubated at 95 degree for 600 seconds and then, a total of 45 cycles were performed at 95 degree for 10 seconds followed by 60 degree for 30 seconds. The results were compared with the control group.

Data analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's HSD using the SPSS 20.0 software. $P<0.05$ was considered as statistically significant difference for all tests.

RESULTS

The SEM images of MET- and IRI-PLGA NPs revealed that the NPs were approximately spherical and in the nano-size range. The mean particle sizes and zeta potential values of MET- and IRI-PLGA NPs were 300±5.87 nm and 216±4.48 nm and -0.121±0.26 mV and -16.37±1.86 mV, respectively. The EE% values for MET- and IRI-PLGA NPs were 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, low EE% was observed.

In cell culture studies, voltammetry state change of glutamate transporter activity when exposed to IRI (1 and 10 µgr), MET (0.5, 1, 2 mM), blank PLGA NPs and IRI/MET-PLGA NPs on both cultures were shown in Figure 5. According to our data, glutamate t_{80} in neuron and U87MG control group was 3 and 86 seconds, respectively. It indicates that glutamate was rapidly re-uptaken by neurons in comparison to GBM cells. In addition, our data showed that blank PLGA did not change t_{80} meaningfully compared with control groups. Free IRI at both concentrations decreased t_{80} nearly four times in GBM culture compared to control group ($P<0.001$) but t_{80} in neuron culture only at 10 µg showed significance difference ($P<0.05$). Free MET reduced t_{80} concentration dependently from 35.2 to 24 in GBM culture ($P<0.001$) but t_{80} culture containing MET (2 mM)

Table 1. T80 time of voltammetry result of GBM and Neuron cell culture

	U87MG				Neuron			
	Mean	±	st.d		Mean	±	st.d	
Control Group	86	±	8,49		3	±	0,230	
Blank PLGA	78	±	2,43		3,1	±	0,010	
İrinotecan 1 µgr	23,5	±	2,79	**	2,5	±	0,013	
İrinotecan 10 µgr	21	±	2,51	**	1,7	±	0,015	*
Metformin 2 mM	24	±	0,73	**	1,8	±	0,009	*
Metformin 1 mM	29,28	±	2,91	**	2,8	±	0,010	
Metformin 0.5 mM	35,2	±	2,24	**	3,3	±	0,030	
İrinotecan PLGA 1 µgr	23	±	0,23	**	2,4	±	0,090	
İrinotecan PLGA 10 µgr	7,1	±	0,12	**	0,7	±	0,004	**
Metformin PLGA 2 mM	11,14	±	1,72	**	0,5	±	0,002	**
Metformin PLGA 1 mM	21,38	±	1,72	**	1,5	±	0,001	*
Metformin PLGA 0.5 mM	28,5	±	1,02	**	2,75	±	0,004	

**P<0.05, **P<0.001*

Table 2. EAAT1 glutamate transporter expression level (mean±St.d) of GBM and Neuron cell culture

	Neuron Culture				U87MG Cell Line			
	SLC1A 2c				SLC1A 2c			
	Mean		st.d		Mean		st.d	
Control Group	1,000	±	0,058		1,000	±	0,347	
Blank PLGA	0,96	±	0,050		1,030	±	0,490	
İrinotecan 1 µgr	0,023	±	0,001	**	49,641	±	1,765	**
İrinotecan 10 µgr	0,012	±	0,000	**	64,737	±	6,280	**
Metformin 2 mM	0,027	±	0,000	**	151,364	±	14,868	**
Metformin 1 mM	0,586	±	0,701	*	27,395	±	0,996	**
Metformin 0.5 mM	0,023	±	0,001	**	18,451	±	0,860	**
İrinotecan PLGA 1 µgr	0,379	±	0,107	**	4,750	±	0,599	
İrinotecan PLGA 10 µgr	0,034	±	0,000	**	24,002	±	0,721	**
Metformin PLGA 2 mM	0,041	±	0,000	**	24,193	±	0,996	**
Metformin PLGA 1 mM	0,011	±	0,000	**	12,901	±	0,860	**
Metformin PLGA 0.5 mM	0,559	±	0,116	**	8,119	±	0,476	**

**P<0.05, **P<0.001*

exhibited significant difference with control group (*P*<0.05). IRI/MET PLGA NPs groups decreased t_{80} more effectively than free drugs especially at higher concentration of both IRI and MET (7.1 and 11.14, respectively) (*P*<0.001). However, in neuron cells, MET-PLGA NPs (2 Mm) were more effective

than IRI-PLGA NPs (10 µg) (P<0.001). In addition, low concentration of MET-PLGA NPs did not decrease t_{80} time in neuron cells. It is obvious that both IRA and MET were more effective on U87MG than on neurons (table 1).

Glutamate transporter expression level was shown in table2. The control group value was set to 1 and all other values were reported according to control group. Blank PLGA EAAT1 expression were close to control group and did not show statically difference in comparison to control group. Free IRI groups decreased transporter expression levels nearly 4 times compared to neurons control group (P<0.001). In GBM culture, transporter gene expression increased up to 49 and 64-fold in a dose-dependent manner compared to control groups. It indicated that the transporter level increased and GBM cells now were able to re-uptake glutamate from the environment. Free MET also decreased gene expression level in neurons but not as effective as free IRI group in neuron cell culture. Free MET effectively and dose-dependently increased transporter expression level up to 151 folds. The gene expression level of IRI-PLGA NPs were similar to free drugs in neurons but in GBM culture, expression level were low compared to free IRI. These data needs to be more investigated. When we look at voltammetry results, time dependent IRI release from vehicle showed glutamate re-uptake t_{80} increased in GBM cancer cells. MET-PLGA NPs increase the t_{80} in GBM cancer cells dose dependently up to 24 folds.

DISCUSSION

Drug-loaded NPs can effectively control the cancer cell proliferation and distribution profile by decreasing drug dosage thereby enhancing the antitumor efficacy [26]. In current study, we investigated the effects of MET- and IRI-loaded PLGA NPs on neuron and U87MG cultures. For this aim, firstly, MET- and IRI-loaded PLGA NPs were prepared and characterized. Then, voltammetry and gene expression level were evaluated in cells incubated with either MEt or IRI after 24 hr.

MET is a cytostatic agent activating AMP-kinase (AMPK) leading to inhibition of the mTOR-signalling pathway (28). Recently, it was suggested that MET can directly reduce glutamate toxicity effect. Because of these reports, Zhou et al. (29) showed that metformin greatly enhanced cell viability against glutamate-induced neurotoxicity. In addition, according to their study, metformin significantly attenuated neuronal apoptosis in glutamate-treated cerebellar granule neurons not only by reducing cytochrome c release and caspase-3 activation but also by phosphorylation of MAP kinases. Our results suggested that metformin was able to directly inhibit glutamate induced excitotoxicity in neurons by reducing EAAT1 transporter and increasing EAAT1 in GBM to increase cell susceptibility to glutamate. However, our voltammetry results showed that transporter activity and t_{80} period after MET administration significantly changed. At the same time, MET was able to inhibit migration of U-87 MG cells because of invasive behavior of GBM and mainly their uncontrolled cellular proliferation (30). On the other hand, IRI, is an important drug for cancer therapy, especially, in combination with other types of chemotherapy agents (31). IRI firstly, was used for the treatment of small and non-small cell lung, ovarian and cervical cancers in Japan (32). After two years, it was approved for the treatment of metastatic colorectal cancer in the United States (33). Animal studies showed that IRI has a significant anticancer activity against an extensive panel of subcutaneous and intracranial human GBM, ependymoma, and medulloblastoma xenografts (34). Likewise, Friedman et al. (35) reported that IRI has favorable activity in adult patients with recurrent malignant glioma.

This data has correlated with the finding of the current study. IRI NPs significantly increased the expression level of glutamate transporter close to 151 folds and glutamate excitotoxicity induced apoptosis and death of GBM cells. In addition, IRI NPs significantly decreased the level of transporter expression compared to control group which indicated that glutamate uptake by neuron decreased.

CONCLUSION

In current study, metformin has direct effect on glutamate re-uptake similar to irinotecan but the effect of metformin on neurons is significantly higher than that of irinotecan. We suggest that metformin and irinotecan can be used in GBM patients.

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ETHICAL APPROVAL

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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