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Target-specific delivery of doxorubicin to human glioblastoma cell line via ssDNA aptamer

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Targeted drug delivery approaches have been implementing significant therapeutic gain for cancer treatment since last decades. Aptamers are one of the mostly used and highly selective targeting agents for cancer cells. Herein, we address a nano-sized targeted drug delivery approach adorned with A-172 glioblastoma cell-line-specific single stranded DNA (ssDNA) aptamer in which the chemotherapeutic agent Doxorubicin (DOX) had been conjugated. DNA aptamer, GMT-3, was previously selected for specific recognition of glioblastoma and represented many advantageous characteristics for drug targeting purposes. Flow cytometry analysis proved the binding efficiency of the specific aptamer to tumour cell lines. Cell-type-specific toxicity of GMT-3:DOX complex was showed by XTT assay and terminated cytotoxic effects were screened for both target cell and a control breast cancer cell line. The result of this contribution demonstrated the potential utility of GMT-3 aptamer-mediated therapeutic drug transportation in the treatment of gliomas specifically. It was concluded that aptamer-mediated drug delivery can be applied successfully for clinical use.

Keywords. A-172; aptamer; doxorubicin; glioblastoma; GMT-3; XTT

1. Introduction

Cancer remains to be one of the major threats for survival on a global basis. It is the second prominent cause of mortality in high-income countries and the number of sufferers is estimated to reach nearly 21 million by 2030 (Bray *et al.* 2012; American Cancer Society 2015). There are different endogenous and exogenous factors that cause cancer such as genetic background, life style and living conditions. These risk factors can act together or in sequence to show their effects (American Cancer Society 2016). Cancerous cells no longer perceive the signals that regulate cell proliferation, differentiation and survival. Persistent angiogenesis and inhibited apoptosis of these abnormal cells can initiate metastasis into distant tissues and even results in death of the organism. Chemotherapy is now the most widely accepted conventional strategy for cancer treatment despite the drugs' undesirable side effects (King *et al.* 2001; Chon *et al.* 2012; Qin *et al.* 2017). The conventional ways of cytotoxic action does not differentiate between healthy and cancerous cells (Gregoriadis *et al.* 1974). In addition, these drugs have drawbacks like poor bioavailability and solubility, rapid blood clearance and nonspecific dispersion (Cho *et al.* 2008; Han *et al.* 2013). Therefore, due to these drawbacks, targeted and controlled delivery of chemotherapeutic drugs has great value to enhance efficiency and reduce unspecific toxicity of those anticancer applications (Krukiewicz and Zak 2016).

Glioblastoma multiforme, a high-grade malignant glioma, is the most frequently confronted tumour of the central nervous system with aggressive progression (Sehedic *et al.* 2015; Karim *et al.* 2016). Glioblastoma multiforme tumours show multifarious histopathological features like necrosis and haemorrhage, microvascular proliferation and cellular polymorphism. Genetic multifarious nature of the tumours including diverse deletions and various point mutations which accelerates the activation of signal transduction pathways have been also accepted as typical (Holland 2000; Hou *et al.* 2006). Although approved treatment consisting of

surgical resection of the tumoural structure prior to radiotherapy and chemotherapy has been applied for patients, only 3–5 % of 2 year survival rate is achieved (Adamson *et al.* 2009). Poor prognosis is mainly related with the recurrent nature of the disease. Complex character of the tumour itself, which has been expressed with the word 'multiforme', is one of the reasons of resistance to therapy, highly recurrent nature and gloomy survival rates.

Nano-sized solutions offer promising targeted drug-delivery advantages to cope up with the complicated histopathology of tumours. They also empower cellular uptake, accumulation and bio-availability of drugs (Peer *et al.* 2007; Bamrungsap *et al.* 2012). Affinity molecules such as aptamers, peptides or antibodies which bind to antigens, receptors or any 3-D structure on the target cells are locomotives for active drug targeting and delivery processes. Due to its nonimmunogenic characteristics, simple chemical synthesis, smaller size, easy acceptance of conjugates, high biological affinity and stability, aptamers have been mostly preferred as specific agents in targeted drug-delivery studies (Sun *et al.* 2014).

Therapeutic agents can be covalently or noncovalently conjugated to aptamer sequences. Being one of those agents, doxorubicin inhibits cancerous cells proliferation via its intercalation into the genetic material mainly at GC-rich sites. Taking advantage of this tendency, DOX can be noncovalently conjugated to aptamers via a simple incubation step with intercalation effect (Akkus *et al.* 2016; Silva *et al.* 2017).

In literature there are many aptamers designated to target glioblastoma cells such as U87-MG (Cerchia *et al.* 2009; Camorani *et al.* 2014), A-172 (Bayrac *et al.* 2011), U118 (Kang *et al.* 2012), U251 (Daniels *et al.* 2003), CD133(+) tumour initiating cells (Kim *et al.* 2013) and U87-EGFRvIII (Tan *et al.* 2013). Beside these cell-SELEX derived ones, more aptamers were developed also for highly expressed glioblastoma surface proteins such as nucleolin (Kotula *et al.* 2012) and EGFR (Li *et al.* 2011). Although there are more than 25 glioblastoma specific aptamers with low K_d values, only few of these aptamers were used in targeted drug delivery studies so far (Delač *et al.* 2015). AS1411 is used for the delivery of PEG-PLGA loaded with paclitaxel to C6 rat brain tumour cells by Gao *et al.* and also same aptamer is used for the targeting of adenovirus to human glioblastoma (Chen *et al.* 2013). GMT8 aptamer is used for the delivery of docetaxel to U87 spheroids (Gao *et al.* 2012a, b), Gint4.T aptamer which recognizes platelet-derived growth factor receptor is used for the delivery of polymeric nanoparticle to U87-MG glioblastoma cells (Monaco *et al.* 2017). Furthermore, Zhang *et al.* used aptamer32 to deliver a siRNA to glioblastoma cells. In our study, we established a glioblastoma targeted delivery system using GMT-3 aptamers as the targeting ligand and this contribution demonstrated the potential utility of GMT-3:DOX complex mediated therapeutic drug transportation in the treatment of gliomas specifically. GMT-3 is an aptamer developed by cell-SELEX specifically for glioblastoma multiforme A-172 cell line. Although developed for one cell line, GMT-3 aptamer can recognize many glioblastoma cell lines with high affinity (K_d ~75 nM) and differentiates them from other cell lines (Bayrac *et al.* 2011). Therefore, we have molecularly assembled DOX into our GMT-3 aptamer to demonstrate the feasibility of use in drug delivery to glioblastoma.

2. Methods

2.1 *Reagents*

All chemicals used in experiments were analytical grade and purchased from Sigma-Aldrich unless otherwise specified. Washing buffer used in the study was composed of 4.5 g/L glucose, 5 mM MgCl2 in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$.

2.2 *Cell lines and cell culture*

Cell line A-172 (ATCC.CRL-1620^{TM}, brain, glioblastoma) was grown in Dulbecco's modified Eagle medium (DMEM, ATCC.30-2002). The MCF-7 (ATCC.HTB-22^{TM}, mammary gland, adenocarcinoma) cells were grown in Eagle's minimum essential medium (EMEM, ATCC.30-2003). All cell lines were supplemented with 10% fetal bovine serum (FBS, GIBCO.26140079) and 100 units/mL of penicillin-streptomycin (Cellgro.30-002CI). Cells were grown in 25 cm^2 or 75 cm^2, filter cap culture flasks (Greiner Bio-One, Germany). When the confluency of attached cells reached to 80% of the flask, surface cells were trypsinated with 2 mL of 0.05% trypsin/0.53 mM EDTA in Hank's balanced salt solution (HBSS).

2.3 *Synthesis of aptamers*

GMT-3 (5′-TGACGAGCCCAAGTTACCTAAGGGTATAC CCCGGGAGGGTGCCA GGTGACCGCACAAGAATCT CCGCTGCCTACA-3′) and tdo-5 (5′-ACCGTGGAGGAT AGTTCGGTGGCTGTTCAGGGTCTCCTCCACGGT-3′) aptamers were synthesized by standard phosphoramidite chemistry using an ABI 3400 DNA synthesizer (Applied Biosystem Inc., Foster City, CA, USA). For the purification of synthesized aptamers reverse-phase HPLC was used with a C18 column (250 mm × 4.6 mm, 5 µm particle size) and a gradient mobile-phase mixture of acetonitrile and aqueous 0.1 M triethyl ammonium amine. Purified DNA aptamers were precipitated with 3 M of sodium chloride solution and cold ethanol at −80°C for

10 min. After centrifugation at 14000 rpm for 20 min pellet was dried with speed vacuum for 5 min. The stock library was resuspended in sterile distilled water and stored at −20°C.

2.4 *Binding of DOX to aptamers*

A physical complex between GMT-3, tdo-5 aptamers and DOX was made by sequential addition of aptamer to DOX. DOX at final concentration of 1.5 µM was incubated with 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0.0781 µM of tdo-5 or GMT-3 for 30 min in washing buffer. Fluorescence was recorded at 480 nm excitation and in the interval of 500–750 nm emission using Nanodrop 3300 (Thermo Fisher Scientific, Massachusetts, USA). The amount of DOX that can physically conjugate throughout the intercalation was determined by the quenching of the DOX. The maximum emission data were recorded at 595 nm and used in the calculation of binding.

2.5 *Biostability of aptamer–DOX complex in cell culture*

Biostability of the aptamer–DOX complex in biological fluids was determined by incubating the complex (1:1.20 mole ratio of aptamer to DOX, DOX was 10 µM) in 10% FBS containing DMEM cell culture media for 2 to 8 hours. After incubation the integrity of the aptamer was visualized by 3% agarose gel electrophoresis.

2.6 *Flow cytometry analysis*

Flow cytometry was used to monitor binding of aptamers to target cells. The biotin-labelled GMT-3 and tdo-5 aptamers were incubated with 5×10^5 target cells at a final concentration of 250 nM on ice for 30 min. The cells were washed twice using 500 µL of washing buffer and then incubated with 200 µL of streptavidin R-Phycoerythrin on ice for 20 minutes. Lastly, cells were washed with 1 mL of washing buffer. The fluorescence intensity was determined using BD Accuri™ C6 Flow Cytometer (Becton, Dickinson and Company, New Jersey, USA) by counting 20000 events.

2.7 *Application of drug and XTT cell viability assay*

A-172 glioblastoma and MCF-7 breast cancer cell lines were used as target and control cell lines, respectively. Briefly, 100 µL aliquot of A-172 and MCF-7 cells (10×10^3 cells/well) were seeded in 96 well plates (n = 4) and allowed to grow overnight. Then, they were treated with either GMT-3:DOX conjugate (1:1.20 mole ratio of aptamer to DOX, DOX was 10 µM), tdo-5:DOX conjugate (1:0.6 mole ratio of aptamer to DOX, DOX was 10 µM) or DOX alone (10 µM) for 45 minutes. After incubation, medium was discarded and each well was washed with 250 µL of washing buffer. After discarding the washing buffer each well was supplied with non-phenolic medium and incubated for 8 hours. XTT-based in vitro toxicology assay kit was used to determine cell viability. XTT solution containing PMSF at concentration of 20% (v/v) was added to the medium. The absorbance was measured at 450 and 690 nm in a microplate reader. The specific absorbance was calculated using the formula:

$$\text{Specific absorbance} = A_{450\text{nm}}(\text{Test}) - A_{450\text{nm}}(\text{Blank}) - A_{690\text{nm}}(\text{Test})$$

3. Results

3.1 *Binding of DOX to aptamers*

DOX is a reputable anticarcinogenic drug which is known to intercalate within DNA strands due to the presence of flat aromatic rings of the molecule (Bagalkot *et al.* 2006). DOX intercalates into double stranded sites and form a physical noncovalent interaction (Min *et al.* 2011). Since each aptamer has a unique shape with different double stranded regions, intercalation efficiency of DOX into the aptamers differs with the sequence identity of those aptamers. Tertiary structures of GMT-3 and tdo-5 drawn by NUPACK were demonstrated in figure 1 (Zadeh *et al.* 2011). GMT-3 aptamer has a strong binding affinity on the A-172 whereas no binding affinity on MCF-7. Tdo-5 was used as a control aptamer and has no binding affinity on both cell lines.

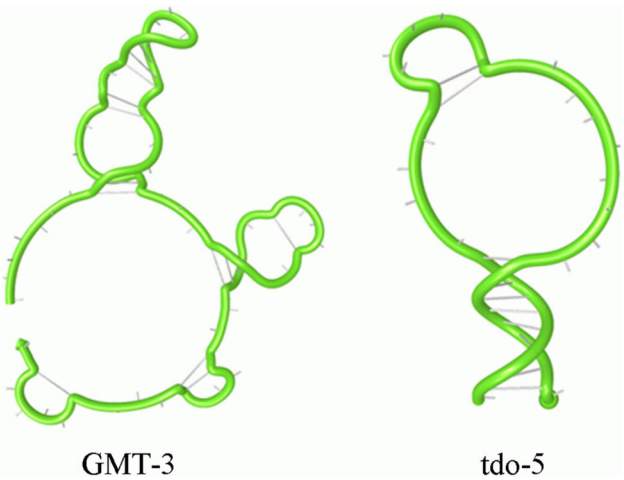


Figure 1. Tertiary structure of GMT-3 and tdo-5 aptamer.

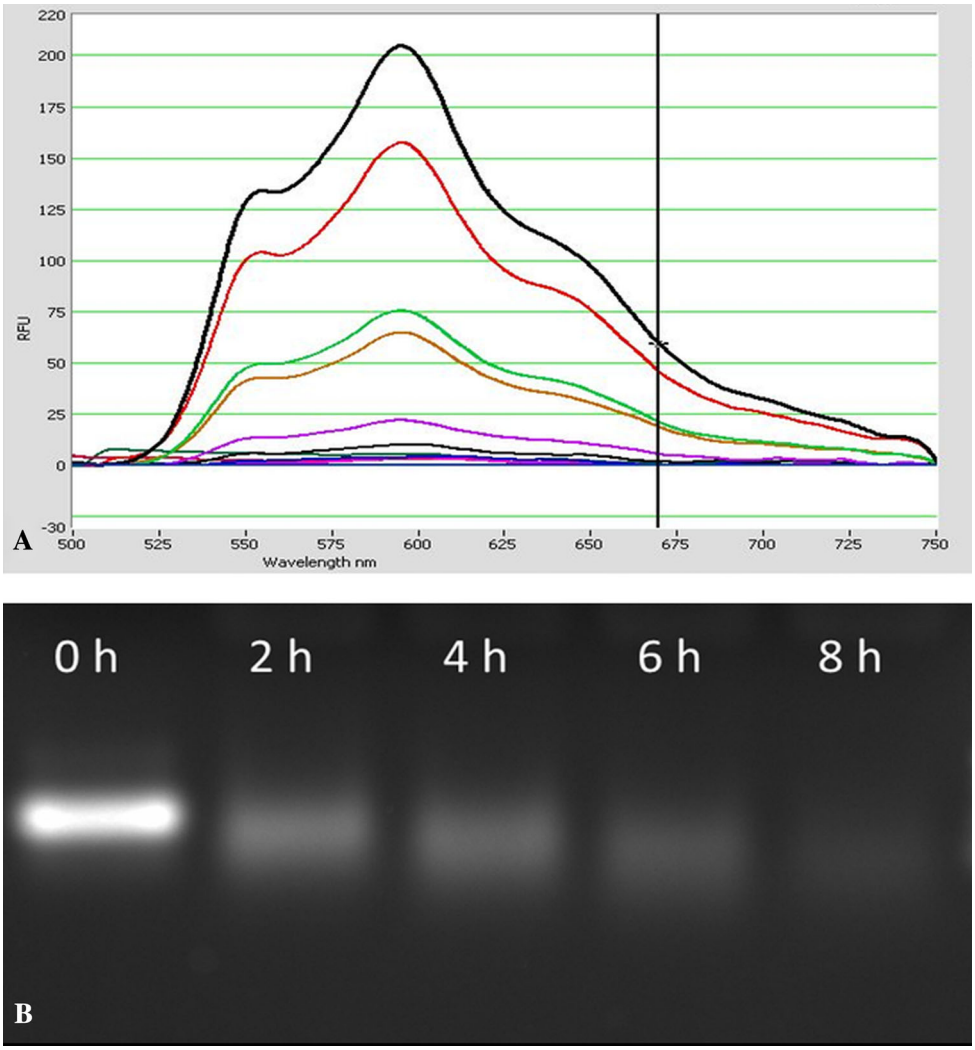


Figure 2. (**A**) Fluorescence spectra of doxorubicin solution with increasing molar ratios of the GMT-3 and (**B**) biostability of aptamer-DOX complex.

Binding of DOX to different aptamers was designated using the fluorescence of DOX molecule. Binding of DOX to aptamers causes a quenching because of the close interaction of aromatic ring. In figure 2A, spectra of fluorescence quenching as a function of increasing aptamer concentration was seen. The relative fluorescence unit (RFU) decreased with the increase in aptamer concentration which implied the positive correlation between aptamer and quenching amount. Table 1 represented the maximum RFU value at 595 nm for GMT-3 and tdo-5 and they were used to form fluorescence quenching graphics of GMT-3 and tdo-5 (figure 3). It is obvious that maximum quenching of 1.5 μM DOX was achieved by the application of 1.25 μM of GMT-3 and 2.5 μM of tdo-5. Based on these results, we used 1:1.20

Table 1. RFU of GMT-3 and tdo-5 with doxorubicin at different concentrations of aptamer at 595 nm

GMT-3 Concentration (μM)	RFU	tdo-5 Concentration (μM)	RFU
0	204.90	0	306.60
0.03	157.50	0.03	249.50
0.07	75.40	0.07	234.00
0.15	64.80	0.15	191.75
0.31	22.00	0.31	114.25
0.62	10.10	0.62	90.90
1.25	3.90	1.25	63.30
2.50	3.60	2.50	37.60
5.00	3.30	5.00	14.80
10.00	5.50	10.00	12.55

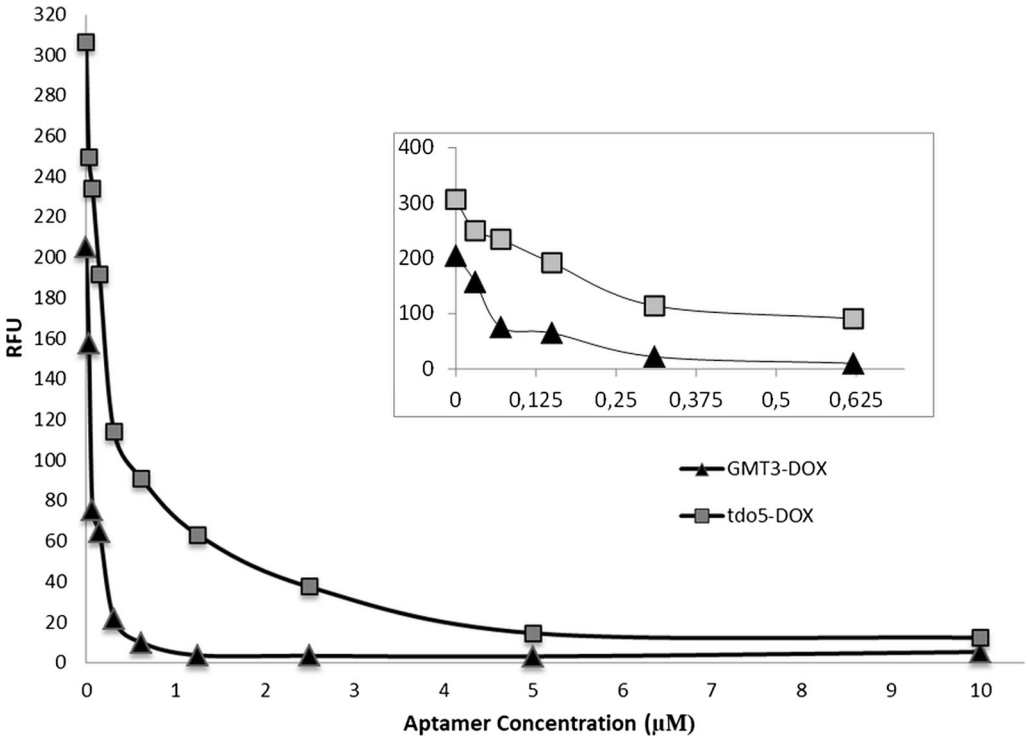


Figure 3. RFU of DOX with different concentrations of GMT-3 and tdo-5.

molar ratio of GMT-3:DOX and 1:0.6 mole ratio of tdo-5:DOX.

Biostability of GMT-3:DOX in cell culture media was determined by incubating the complex in cell culture media. The aptamer degradation was increased by the increase in incubation time and bands in agarose gel became smeary (figure 2B).

3.2 *Binding of aptamers to target cells*

Flow cytometry was used in order to show the specific binding of aptamers to the cell lines. In figure 4, it was clearly seen that GMT-3 was binding to A-172 glioblastoma cell line and causing a significant shift in the logarithmic fluorescence axis but it was not binding to MCF-7 breast cancer cell line. Beside, control aptamer tdo-5 did not show any noticeable binding to both cell lines.

3.3 *Specific growth inhibition of DOX bound aptamers*

After showing specific binding of aptamer to its target cell, we next examined the effect of targeted delivery of the aptamer-DOX physical conjugate on cellular toxicity. It was demonstrated that the targeted delivery of the conjugate results in augmented cellular cytotoxicity as compared to untreated cells. Same dose of DOX loaded on the aptamers were applied on both the target cell line A-172 and control cell line MCF-7. The data represented that GMT-3:DOX conjugate caused more inhibition on growth of target cells A-172 (41.30% ± 3.75%) than the control cell line MCF-7 (82.13% ± 4.68%) (figure 5). The application of only DOX at same dose was causing cytotoxicity in both cell lines with viability of 39.94% ± 6.34% for A-172 and 38.27% ± 2.87% for MCF-7. Besides, tdo-5:DOX conjugate was not causing apparent cytotoxicity in both cell lines. DOX delivered with tdo-5 decreased viability of A-172 cell lines to 89.20% ± 7.00% and MCF-7 cell lines to 87.71 % ± 6.41%.

4. Discussion

Despite the progress in detection technologies and high numbers of ongoing researches, many cancer types still do not have effective treatments. Due to its high implementability, chemotherapy is the best alternative among various therapeutics for the treatment of cancer (Pasquier *et al.* 2010). Although it is highly efficient, common and practicable, chemotherapy has the problems of nonspecific distribution and poor bioavailability. Thus, finding new strategies for delivering chemotherapeutics to target site is important to improve treatment (Qin *et al.* 2017). Targeted drug delivery is the one of the hottest prospect for the future in cancer therapy because of the toxicity of the chemotherapeutic drugs that fails to act specifically on cancer cells and give damage to healthy

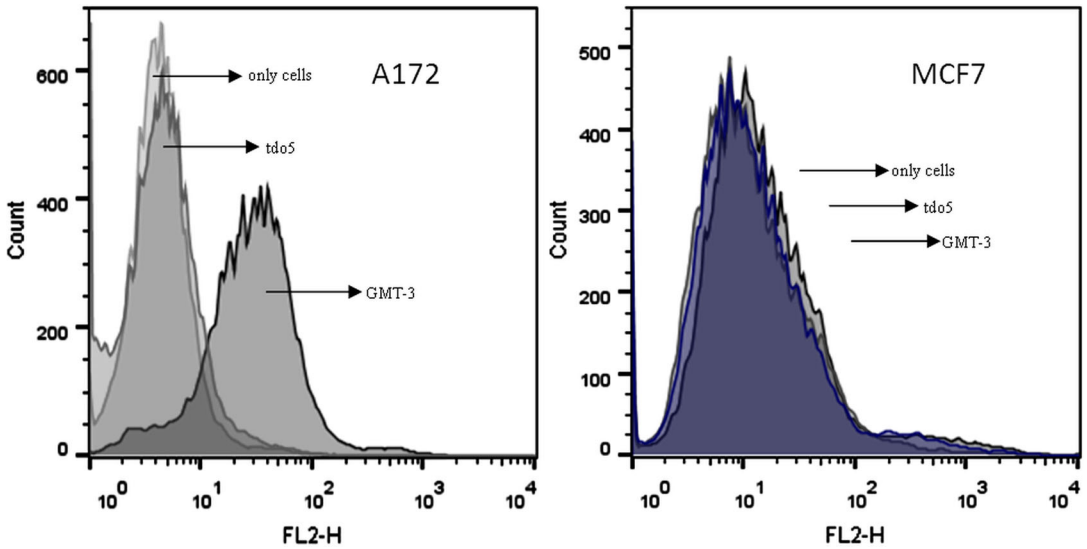
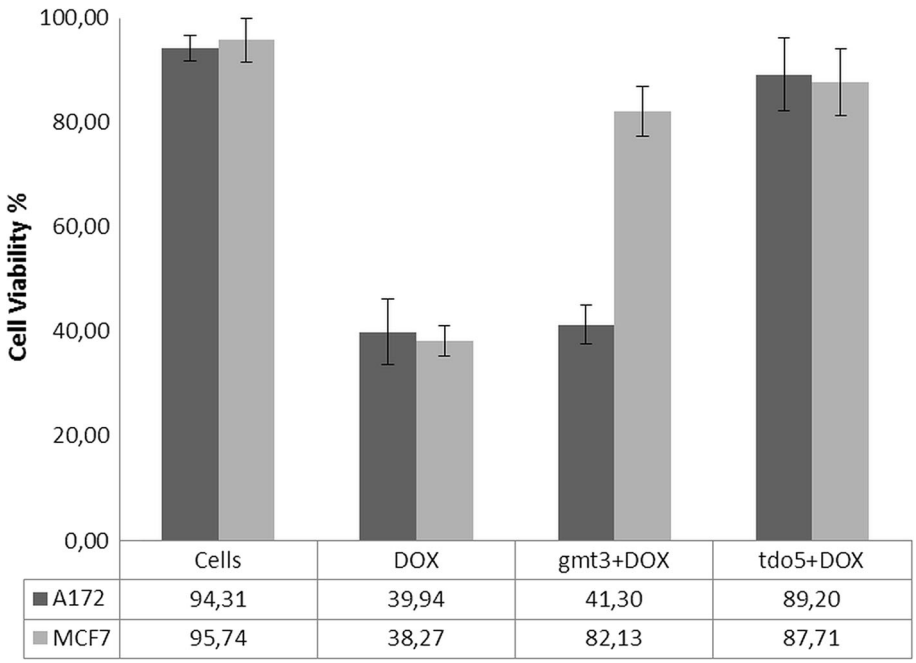


Figure 4. Flow cytometry assay for binding of GMT-3 and tdo-5 aptamers to A-172 and MCF-7 cell lines.



	Cells	DOX	gmt3+DOX	tdo5+DOX
A172	94,31	39,94	41,30	89,20
MCF7	95,74	38,27	82,13	87,71

Figure 5. Effects of DOX, GMT-3:DOX and tdo-5:DOX complex on A-172 and MCF-7 cells viability (XTT assay).

cells (Li *et al.* 2014). DOX has been one of the mostly utilized drugs worldwide for many years but its high toxicity even after treatment limits its usage (Meng *et al.* 2012, Meredith *et al.* 2016).

Aptamers are synthetic single-stranded oligonucleotides that bind target molecules with high specificity and affinity. Many aptamers are developed for the recognition of cancer cells and used in various platforms for detection and targeted delivery (Hernandez *et al.* 2013). In this study, we used GMT-3 aptamer which was developed specifically for glioblastoma multiforme (Bayrac *et al.* 2011) and tdo-5 aptamer as a control which was specifically developed for B-cell lymphoma (Tang *et al.* 2007). GMT-3 and tdo-5 aptamers formed a complex with DOX in different ratios because of the difference in their 3-D structure (figure 1). Since DOX interacts with DNA by simple intercalation and DOX-DOX self-association is enhanced along the double helix (Silva *et al.* 2017) different sequences of DNA has different DNA:DOX complex ratios. Intercalation of DOX was evaluated by the reduction of DOX fluorescence intensity (figures 2A and 3) Maximum

aptamer:DOX ratios were found to be 1:1.20 molar ratio of GMT-3:DOX and 1:0.6 molar ratio of tdo-5:DOX. This result was also concordant with the high number of double helixes present in the GMT-3.

In order to show the binding efficiencies of the aptamers flow cytometry was used. Flow cytometry analysis showed that A-172 cell line had significantly higher fluorescence intensity when incubated with GMT-3 as compared to incubation with tdo-5. On the other hand, both GMT-3 and tdo-5 did not change the fluorescence intensity when incubated with MCF-7 cell. This concluded that GMT-3 was binding to a surface molecule of A-172 cell line specifically but not to MCF-7 cell line. Beside, tdo-5 aptamer did not show any binding affinity to both A-172 and MCF-7 cell lines (figure 4). These binding patterns made them feasible candidates to test targeted drug delivery. After complexing with DOX, only GMT-3 aptamer was expected to deliver its cargo solely to A-172 cell line. Moreover, MCF-7 cell line should not be affected by GMT-3:DOX complex and tdo-5 should not be able to deliver DOX to both A-172 and MCF-7 cell lines. Aptamers can deliver the cargo to target cells but they should also release the drug or be internalized by the cell in order to let the drug to show its effect. In order to demonstrate this phenomenon we mimicked the degradation of oligonucleotide in cell culture conditions. As shown in figure 2B, aptamer carrying the DOX was degraded by the nucleases of FBS present in the cell culture media. While the degradation was intense after 4 h of incubation, integrity of the aptamer was totally lost with no visible DNA band on the gel upon 8 h of incubation. This showed that nucleases present in the cell culture began degradation instantly but it took time to degrade all DNA. Although it is compulsory to release the drug, degradation of the aptamers is a main drawback for targeted delivery. Since there are many nucleases in blood, many of the aptamers can be degraded throughout the circulation before reaching the target site. Since glioblastoma multiforme is an intracranial cancer, beside intravenous application drug can be directly applied through cerebrospinal fluid (CSF). Unlike blood, CSF has less DNase activity and unmodified oligonucleotides remain undegraded up to 24 h at 37°C in CSF (Whitesell *et al*. 1993; Hughes *et al.* 2009). All the same time, modification of aptamer sequences with protective groups such as polyethylene glycol, 2′-fluoropyrimidine or 2′-*O*-methylpurine can easily make them suitable for intravenous application and prolong their half-life in biological fluids (Healy *et al.* 2004).

XTT assay confirmed our binding data and tdo-5:DOX complex did not have any significant effect on the viability of A-172 and MCF-7 cell lines. On the other hand, GMT-3:DOX complex significantly decreased the viability of its target cell line A-172 ($p<0.05$) while not affecting the MCF-7 cell line. Because of its non-targeted nature, only DOX application decreased the viability in both cell lines (figure 5). This assay indicated that GMT-3 aptamer easily differentiated the cell type and target cargo to its destination.

5. Conclusion

GMT-3 aptamer can deliver noncovalently conjugated DOX to glioblastoma cell line and release the drug causing cytotoxicity while not affecting the non-target cell lines because of the specific binding. This system can reduce the cytotoxic effects of chemotherapeutics making it applicable delivery system for clinical use.

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