Original Paper

The Potential of Helianthin Loaded Into Magnetic Nanoparticles to Induce Cytotoxicity in Glioblastoma Cells

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ABSTRACT: The central nervous system tumors are the most common solid tumors in adults.. Unlike other types of cancers, brain cancer is much difficult to treat because of the blood-brain barrier (BBB) that prevents drug substances from crossing it and accessing the brain. Different types of methods to overcome BBB have been used in vivo and in vitro, of which the use of nanoparticle-mediated delivery of therapeutic drugs is particularly promising. In the present study, we used iron oxide magnetic nanoparticles (NPs) as carrier system for helianthin (He/NPs) to treat cancer cells derived from glioblastoma. An early passage cell cultures (GB1B), established in our laboratory from tissue obtained from a patient diagnosed with glioblastoma, was used. The cells were treated with different concentrations of NPs or HeNPs and then cell proliferation was measured at 24, 48 and 72 hours. Our results showed that the treatment with NPs was well tolerated by glioblastoma cells, the viability of the cells increased very slightly after the treatment. Furthermore, we demonstrated that helianthin loaded Fe3O4 magnetic nanoparticles induced cytotoxicity in human glioblastoma cells. The treatment with HeNPs induced dose and time dependent.

KEYWORDS: *Glioblastoma; helianthin; magnetic nanoparticles.*

Introduction

Recent medical development has improved outcomes for patients diagnosed with a variety of malignant diseases.

However, brain cancer management is still challenging, despite of the research and innovation efforts [1].

Many circumstances are suggested to make glioblastoma (GB) more difficult to treat, compared to other types of cancers:

I) even if GBs treatment requires surgery as the first line of intervention, the location of the tumors in critical areas of the brain represents a serious impediment;

II) II) GB cells proliferate quickly and invade neighboring normal brain tissue, resulting in poor prognosis and rapid recurrence;

III) III) the existence of heterogeneity within and between GB tumors, often disconcerting the clinical intervention;

IV) IV) the chemotherapeutic drugs hindrance to rich the tumor place, by blood-brain barrier (BBB), is another aspect in treatment failure.

In the last decade, *in vitro* and *in vivo* GB research generated huge information about drugs targeting several survival signaling pathways.

Although these molecules showed therapeutic potential against GB cells *in vitro*, their clinical potency in GB patients remains questionable [2].

The main problem for the *in vivo* treatment failure is supposed to be the BBB restriction of the drug to bring effective concentrations at the tumor site [3].

Much effort has been made by scientific community to find BBB-penetrating drugs to treat GB patients.

Nanoparticles drug delivery was reported to be an efficient method to help cytotoxic molecules to perforate through BBB but also to entry in tumor vasculature, tolerating better access to tumor cells [4-6].

An additional ***reason for using*** of nanoparticles as carrier systems for therapeutic drugs is to enhance the therapeutic effect while reducing the drug side effect [7].

Fe_3O_4 magnetic nanoparticles (NPs) are the most conventional magnetic nanoparticles, used in a lot of medical areas, particularly for targeted drug delivery systems in cancer treatment [8-10].

In our previously studies, we found that Helianthin, an azo dyes compound, induced cell death in high grade glioma cells [11].

In this study, we analyzed the effect of Helianthin loaded NPs (HeNPs) on GB cells *in vitro*.

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Material and Methods

Synthesis of Fe3O4/Helianthin magnetic nanoparticles

Analytical grade reagents (Helianthin, KOH, $FeCl_3$ and $FeCl_2$) were obtained from Sigma-Aldrich.

The Fe_3O_4/Helianthin (HeNPs) core-shell magnetic nanoparticles were synthesized by a modified Massart method [12].

Fe_3O_4/salicylic acid Magnetic nanoparticles synthesis and functionalization were previously described in the literature [12,13].

$FeCl_3$ and $FeCl_2$ hydrated salts (2g $FeCl_3$, 1.25g $FeCl_2$) were dissolved in 250ml of ultrapure water and then precipitated under basic condition.

The surplus of KOH and Helianthin was eliminated by several washing steps, involving sonication and magnetic separation [14].

Then, a DLS analysis was used to evaluate Zeta potential and the hydrodynamic diameter of the HeNPs.

In brief, the mean diameter (Z-average) and the polydispersity index (PdI) of the nanoparticles in aqueous dispersion were analyzed at an angle of 173º, with a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom).

All measurements were operated at 25ºC and data were presented as a mean of five separate measurements.

By using DLS analysis, a good dispersions stability was confirmed.

The potential value was 42.2mV, polydispersity index was 0.119, and the hydrodynamic diameters of magnetic dispersions 65.9nm confirmed that the HeNPs dispersions are monodisperse [15].

In vitro assay

GB cell line establishment from glioblastoma tissue

GB1B cell line, was established in our laboratory, according to standard procedures, by using residual biological material (tumour tissue) from a glioblastoma patient, operated at the Emergency Hospital, "Bagdasar-Arseni", Bucharest, Romania.

Briefly, <5mm diameter tumour pieces were mixed with 0.25mg/ml collagenase IV, 0.4mg/ml DNase, and 0.5mg/ml pronase, then the sample was transferred ***in*** a ***shaking incubator*** at 37°C in Hank's buffered saline solution for 30min at 37^0C, followed by 30min at 4^0C.

The cell suspension was passed through a tissue culture sieve and then transferred into tissue culture flasks [16].

Cell treatment

GB1B cells were grown in monolayers in tissue culture flasks, in standard minimum essential medium (MEM) (10% fetal bovine serum, 2mM glutamine and 100UI/ml penicillin/streptomycin, in a humidified incubator (95% air/5% CO_2 atmosphere at 37°C) cells were then grown in 6-well, culture plates, at a of (2,000-3,000 cells/cm^2 density) and experiments were initiated at 50% confluence.

The cells were treated with 0.25µg/ml, 0.5µg/ml, and 1µg/ml Fe_3O_4 NPsor HeNPs and then the sample was incubated for 24, 48 and 72 hours. Control samples with diluents only were included.

Cell viability

Cells were grown in 6 wells plate, exposed to different concentration of the nanoparticles and cell proliferation was measured by determining the number of cells attached to the plastic surface of duplicate wells.

This was performed by microscopic counting of cells in ink-marked areas on the wells bottom.

By repeating the counting's after specified time intervals, changes in the number of attached cells could be followed.

Statistical analysis

The data are shown as the average±SD and were analyzed using two-tailed T-test to examine both sides of a defined data range as designated by the probability distribution involved.

$P<0.05$ values were considered statistically significant.

Results

The effect of NPs on glioblastoma cells

Iron oxide magnetic nanoparticles are commonly used as drug carrier systems in oncological applications, yet questions remain ***regarding*** their cytotoxicity on malignant cells.

Some studies showed that NPs treatment induced a strong cytotoxic effect on cancer cells, while other studies [17] reported weak or no cytotoxicity [18], but there are also studies showing that NPs have no cytotoxic effect on cancer cells [19].

Our results showed that exposure of glioblastoma cells to 0,25µl/ml NPs for 24 and 48 hours induced a slight decrease in GB1B cell

viability but the results were not statistically significant (p>0.05) (Figure 1).

As seen in Figure 1, the 0,25µl/ml NPs treatment for 72h, did not influence the viability of the GB1B cells compared to the untreated cells (p>0.05).

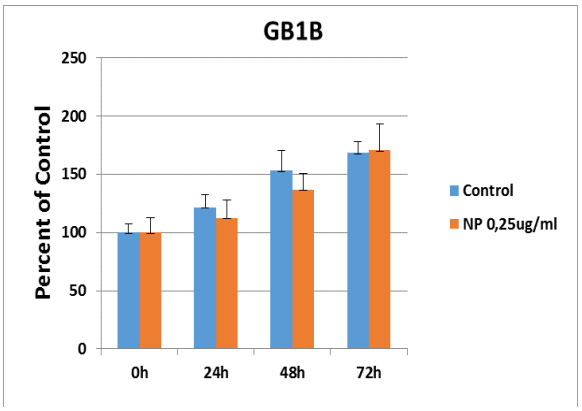

Figure 1. NPs influence on the GB1B cells viability. The treatment effect is expressed as percentage of corresponding control and data are reported as average±SD.

Unexpected, the increase in NPs concentration to 0,5µl/ml induced an increase in GB1B cell proliferation from 122 percent to 141 percent, 24h and after the treatment (p>0.05); from 153 percent to 172 percent, 48h and after the treatment (p>0.05) and from 168 percent to 198 percent, 72h and after the treatment (p>0.05) (Figure 2).

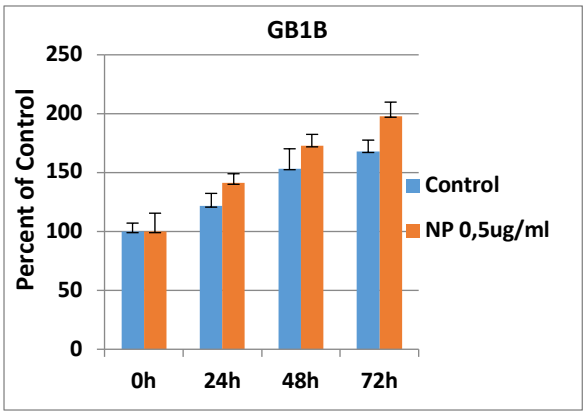

Figure 2. Effect of NPs on the GB1B human glioblastoma cells viability. The cells were treated with 0.5µl/ml NPs for an indicated time. Results are shown as% of control and data are reported as arithmetic mean±SD.

The treatment with 1µl/ml NPs concentration induced an increase in GB1B cell proliferation from 122 percent to 142 percent, 24h and after the treatment (p>0.05); from 153 percent to 164 percent, 48h and after the treatment (p>0.05) and from 168 percent to 207 percent, 72h and after the treatment (p>0.05) (Figure 3).

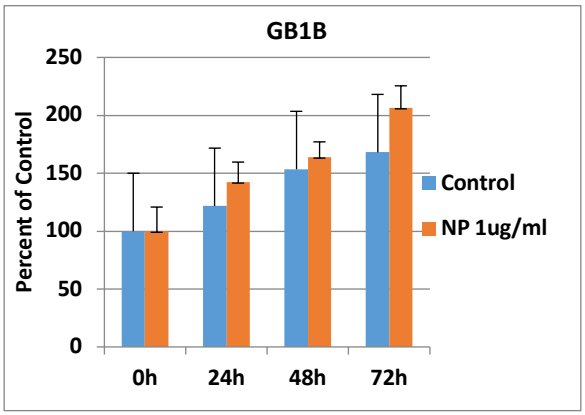

Figure 3. Effect of NPs on the GB1B human glioblastoma cells viability. The cells were treated with 1µl/ml NPs solution and the cell viability was determined by microscopic counting of cells at 24, 48 and 72 hours. Results are expressed as% of control and reported as mean±SD.

Taken together, our results also showed that Fe_3O_4 magnetic nanoparticles were not cytotoxic on glioblastoma cells.

The effect of HeNPs on glioblastoma cells

It is very problematic to design drugs that reach their target cells in the central nervous system, due to their incapability to pass through the BBB.

In this context, nanoparticles have sparked special interest in brain tumors treatment as efficient delivery systems of the chemotherapy drugs into the CSN. In 1996, the Food and Drug Administration (FDA) approved a new drug delivery technique, involving the introduction of nanoparticles (range from 1 to 100nm) such as polymers, nanocrystals, metals/metal oxides, etc. [20].

To avoid air oxidation, iron oxide magnetic nanoparticles are usually functionalized in different way, such as graphene oxide materials or carbon materials different polymeric materials silica, etc. [21-24].

In this study, we used ferrite magnetic nanoparticles (NPs) to carry Helianthin drug, previously showed to kill brain tumor cells (HeNPs).

The next step in our experiment was to observe how HeNPs influence cellular proliferation in the GB1B cell line (Figure 4).

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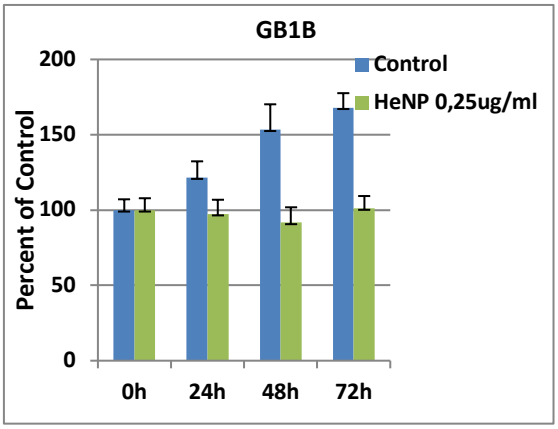

Figure 4. Effect of HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with 0.25µl/ml HeNPs for 24, 48 and 72 hours. Data are expressed as percentage of corresponding control and the experiments were repeated at times. Data are shown as mean±SD.

At 24h, proliferation for the cells treated with HeNPs was already hindered by 24% in comparison to the control group ($p>0.05$).

After 48h, a major difference between the cells exposed to HeNPs versus the untreated cells can be observed: the cells in the control group presented a growth in proliferation by 50% while the treated cells presented a cytotoxicity of 9% ($p<0.05$).

At 72h, cells treated with 0.25µg/ml HeNPs presented a slight increase in proliferation to 101% when compared to the control group ($p<0.05$).

However, a major discrepancy in proliferation, of 67%, between treated and untreated cells can be observed (Figure 4).

When compared, HeNPs have a distinctively stronger effect in contrast to unloaded NPs (Figure 5).

At 24h, HeNPs managed to halt proliferation in the GB1B line while cells treated with NPs presented a 12% growth in proliferation ($p>0.05$).

At 48h, cells treated with HeNPs presented a 47% difference in cytotoxicity when compared to cells which received NPs treatment ($p>0.05$).

After 72h, a stark difference in proliferation, of 70%, was observed between the two treatment options, strongly in favor of HeNPs ($p<0.05$).

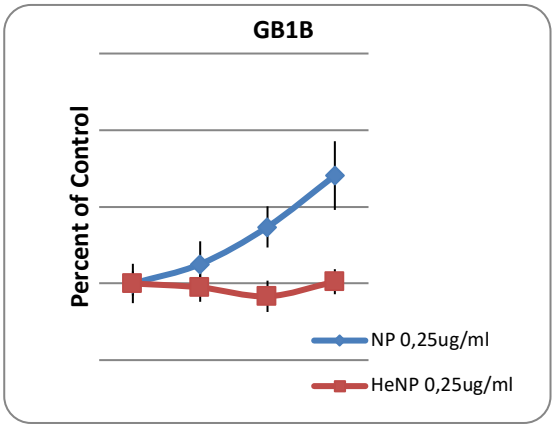

Figure 5. The effect of NPs and HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with 0.25µl/ml NPs or 0.25µl/ml HeNPs solution for 1, 2 and 3 days, and the viability was analyzed by microscopic counting. Results are expressed as percentage of control and the experiments were repeated three times. Data are reported as mean±SD.

We further increased the dose of HeNPs to 0.5µg/ml (Figure 6).

After 24h, the treated succeeded in completely blocking proliferation, presenting a cytotoxic effect of 30% when compared to untreated cells ($p>0.05$).

This trend was maintained throughout the experiment with the cells in the experimental group presenting no changes in their proliferation pattern, strongly contrasting cells in the untreated group ($p<0.05$).

Overall, the difference in proliferation was 30% at 24h, 62% at 48h and 78% at 72h (Figure 6).

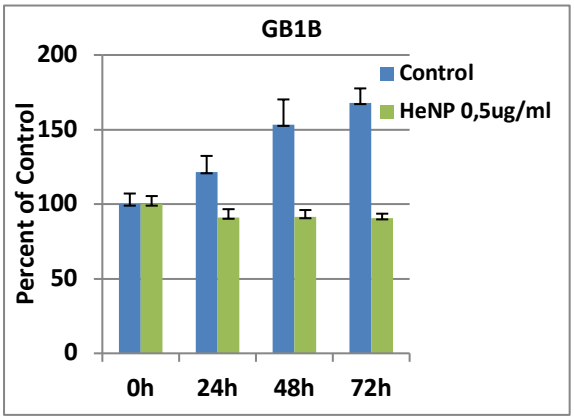

Figure 6. Effect of HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with a solution of 0.5µl/ml HeNPs and then the living cells were microscopic counted at the end of the treatments (24, 48 and 72 hours). All data are presented as percentage of untreated control and reported as mean±SD. The experiments were repeated three times.

As seen in Figure 7, our results showed that the dose of 0.5µg/ml HeNPs presented a markedly superior cytotoxic effect when compared to the 0.5µg/ml dose of unloaded NPs.

At 24h, the difference in proliferation between the unloaded and loaded NPs was 50%, the HeNPs managing to reverse proliferation in the GB1B cell line ($p>0.05$).

After 48h, the difference in proliferation observed between the two treatment options increased, with cells which received only 0.5µg/ml NPs treatment presenting a 75% rise while cells which were exposed to HeNPs presenting no proliferation when compared to the previous time point ($p<0.05$).

After 72h, the number of cells treated with NPs essentially doubled while cells treated with HeNPs continued to exhibit no proliferation ($p>0.05$).

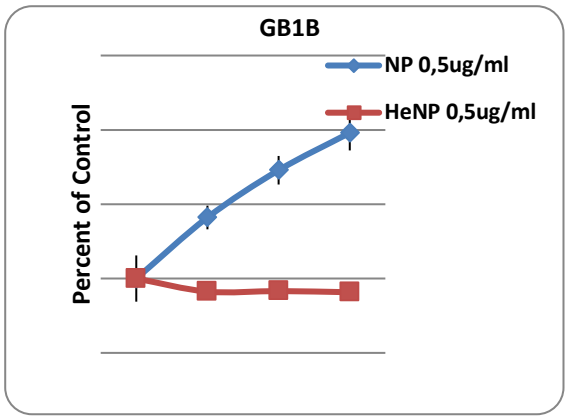

Figure 7. The effect of NPs and HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with 0.5µl/ml NPs or 0.5µl/ml HeNPs mixture for 24, 48 and 72 hours. Cell viability was determined at the end of each experiment and the data are expressed as % of untreated control cells. Data are reported as mean±SD.

For the maximum dose of 1µg/ml HeNPs used in our experiment, the cells presented decreased proliferation in all the time points considered (Figure 8).

After 24h, the 1µg/ml dose managed to reduce proliferation by 30% when compared to untreated cells ($p>0.05$).

The difference between untreated and treated cells continued to grow and after 48h, the untreated cells presenting a 74% more enhanced proliferation compared to their treated counterparts ($p<0.05$).

After 72h, the cell line treated with 1µg/ml HeNPs remained virtually unchanged compared to the untreated cells which continued to proliferate ($p<0.05$).

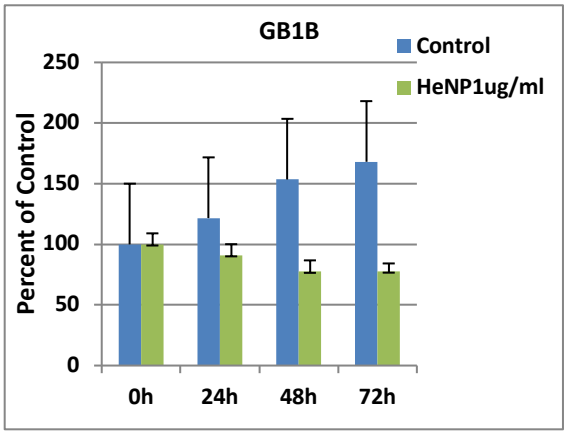

Figure 8. Effect of HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with 1µl/ml HeNPs solution and the percentage of viable cells was determined by microscopic counting at the end of the treatment (24, 48 and 72 hours). Results are expressed as percentage of control and the experiments were repeated three times. Data are reported as mean±SD.

In figure 9, we compared cell responses at treatment with 1µg/ml NPs and 1µg/ml HeNPs over 24, 48 and 72 h.

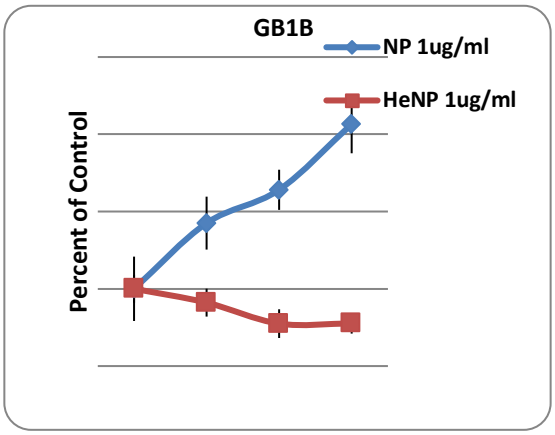

Figure 9. The effect of NPs and HeNPs on the GB1B human glioblastoma cells viability. The cells were treated with 1µl/ml NPs or 1µl/ml HeNPs, cell proliferation was determined by microscopic counting of viable cells at 24, 48 and 72 hours and the results were calculated as percentage of untreated control. Data are reported as mean±SD.

The highest dose of 1µg/ml NPs used in our experiment produced markedly different results when compared with HeNPs, in inducing cytotoxicity in GB1B cells.

After 24h, the difference in proliferation between cells treated with either loaded or unloaded NPs was 51% ($p>0.05$).

At 48h, cells treated with unloaded NPs continued to proliferate while cells which were

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treated with 1μg/ml presented a decrease in proliferation of 23% compared to untreated cells ($p<0.05$).

After 72h, the cells which were treated with unloaded NPs presented a two-fold increase in proliferation while cells treated with 1μg/ml HeNPs presented no change in proliferation when compared to the previous time point ($p<0.05$).

Discussion

Glioblastoma, a grade IV malignant tumor originating from astrocytic cells in brain, is the most lethal form of brain cancer.

Survival of glioblastoma patients is very short, due to lack of efficient therapeutic approaches.

Currently, an oral alkylating chemotherapy compound, namely TMZ, is the preferred drug for high-grade gliomas treatment, reported to produce survival benefits in patients.

Despite TMZ is able to cross the blood-brain barrier, the drug requires repeated administration and it is often necessary to increase the dose of the drug, due to the resistance that the tumor develops to treatment [25].

Unfortunately, a higher dose of TMZ may have severe side effects, such as hepatotoxicity, cardiomyopathy, etc. [26].

These restrictions associated with TMZ therapy, have generated the need to develop new methods that allow for the systemic administration of a higher amount of drug, in order to increase e its therapeutic index.

Polymers and liposomes have been tested as nanocarriers for TMZ administration in glioblastoma treatment, however, the benefit of these delivery systems was modest due to lack of tumor specific delivery of the drug [27].

The accelerated development in nanotechnology resulted in several changes in the field of cancer treatment.

Synthesis of new drugs for targeted therapy as well as their delivery systems, are some of the newest discoveries that provide many benefits in clinical oncology.

Nanoparticle-based cancer management is the key component of nanomedicine, drawing ample interest in both diagnosis and treatment.

However, available data reported in literature concerning the nanoparticles showed that some type of them are toxic, while other categories do not produce cytotoxicity [27-29].

Among various types of nanoparticles, Fe_3O_4 magnetic nanoparticles (NPs) are commonly used in cancer drug delivery treatment, because of their biocompatibility, biodegradability and safeness [28,30,31].

Many studies have demonstrated that Fe_3O_4 degrades into its chemical elements that are then assimilated in human iron metabolism [32].

In this study, we found that the treatment with NPs was well tolerated by glioblastoma cells, inducing increase in cell viability.

Magnetic particles were suggested to be effective for the targeting delivery of antineoplastic drugs to tumor site through the application of a magnetic field.

Thus, NPs drug delivery system was shown to improve therapy effect by accumulating the drug in the tumor, which in turn result in systemic toxicities reduction ,as a result of decreasing in systemic drug concentrations [33].

In a research study published in Scientific Reports in 2017, Sonali Kumari et al. showed that TMZ encapsulated in lactoferrin nanoparticles was efficient in drug delivery through the BBB, resulting in a significant reduction in tumor burden in orthotropic glioma model [34].

Several research studies described enhancement in malignant cellular uptake of cytostatics uploaded in NPs in many types of malignant diseases.

For example, doxorubicin loaded magnetic nanoparticles were very efficient in killing glioma cells [35].

In a Phase I clinical trial conducted by Andreas Stephan Lübbe et al., 14 advanced solid liver cancer patients were subjected to magnetic drug targeting with epirubicin and the results showed that the treatment was well tolerated and nontoxic [36].

We previously found that Helianthin induced cell death in high grade glioma cells [11].

Very little is known about small molecules functionalized NPs effect on cancer cells.

In our previously studies, we found that Helianthin induced cell death in GB cells by downregulation of the EGFR and IGF-1R activities [11].

Conclusions

In the current study, we analyzed to effect of HeNPs in GB1B cells. Our results showed that Helianthin coated NPs were cytotoxic for glioblastoma cells *in vitro***.**

Drug-loaded iron oxide ***nanoparticles*** allowed a decreased drug dosage, minimizing its cytotoxicity and improving BBB penetration of drug.

However, for the application of HeNPs for *in vivo* targeted delivery, more data about the toxicity and the capacity to cross BBB of the HeNPs should be accumulated.

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Alexandra Costachi, Cătălina Elena Cioc, and Sandra Alice Buteică contributed equally to this study.

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Competing interests

There are no competing interests to declare regarding this study.

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