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Metformin delivery using chitosan-capped gold nanoparticles in glioblastoma cell lines

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Abstract: *Introduction*: Metformin (MET), an old anti-diabetic drug, has proven unexpected anti-glioblastoma effects, by impacting cell proliferation, migration and invasion. However, its remarkable anti-cancer efficacy is mainly limited to the use of high millimolar concentrations in in vitro studies, which are hard to be attained in the clinical setting. *Aim*: The aim of this paper was to synthetize gold nanoparticles loaded with MET and to test if an enhanced drug delivery via nanotechnology could overcome the limitations of small drug concentrations. *Materials and Methods*: Gold nanoparticles were functionalized with chitosan (GNPc) and loaded with 80 μM of MET. Their size, zeta potential and stability were characterized and their internalization within tumor cells was assayed through dark field microscopy. Three primary glioblastoma stem cell lines were treated with 5, 10 and 20 μg/mL concentrations of nanoparticles and irradiated. The anti-tumoral effect was evaluated through the MTT cell viability assay. *Results*: MET-GNPc are easily synthetized and have a positive zeta potential, spherical shape and a median size of 26 nm. MET-GNPc have an increased cell internalization and affect the viability of all three glioblastoma cell lines used compared to control and free MET. However, their anti-cancer effect is not statistically different when compared to GNPc, although a slight tendency to a better response may be observed. *Conclusion*:Despite an increased cell internalization, the small micromolar concentrations of metformin does not bring an additional benefit to chitosan-based GNPs. Novel delivery methods being able to carry a higher drug concentration of metformin should be tested.

Introduction

Metformin (MET), a well-known oral antidiabetic, has been regarded as an extremely promising anticancer agent in many cancer types. In vitro and in vivo experiments proved a myriad of mechanisms of action, including decreased cell proliferation, cell cycle arrest, autophagy, apoptosis and cell death in vitro with a concomitant activation of AMPK and inhibition of the mTOR pathway, while also sensitizing cells to radiotherapy (M. Aldea et al., 2014; M. D. Aldea et al., 2014; Carmignani et al., 2014; Nenu et al., 2014; Sesen et al., 2015; Soritau et al., 2011). Our team investigated MET in glioblastoma cell lines and proved a surprising reduction of glioblastoma stem-like cells proliferation (M. D. Aldea et al., 2014; Carmignani et al., 2014; Soritau et al., 2011).

However, this benefits were observed mainly at supra-clinical doses of 1-10 mM, which are easily obtained in vitro or in vivo experiments, but are unlikely to be obtained in the clinical setting (M. Aldea et al., 2014). Clinical cancer trials that have tested MET with the same doses used in anti-diabetic therapy failed to translate into a relevant tumor response, possibly due to lack of MET adequate high concentrations for cancer cell killing (Kordes et al., 2015; Reni et al., 2016; Sayed, Saad, El Wakeel, Elkholy, & Badary, 2015).

We hypothesize that MET tumor targeted delivery by nanotechnology might overcome the ineffectiveness of small drug concentrations. Therefore, in this study, we synthetized gold nanoparticles loaded with micromolar concentrations of MET in order to test if such low dose compounds reach the anticancer effects observed with high free drug MET therapy.

Materials and Methods

Nanoparticles preparation and metformin loading

Citrate-capped gold nanoparticles (GNPc) were synthetized following the Turkevich–Frens method and by slightly modifying the method used in our previous experiments (Aldea M, 2018; Potara, Maniu, & Astilean, 2009; Turkevich J, 1951). Briefly, 100 mL of hydrogen tetrachloroaurate(III) trihydrate HAuCl4·3H2O (10-3 M) were heated to a boiling temperature and then mixed to trisodium citrate (10 mL, 38.8 × 10−3 M) (Merck) under vigorous stirring. After the formation of a deep-red burgundy colloid, the stirring and boiling processed were continued for 10-15 min. Subsequently, the heating was stopped and the stirring process was continued for another 15 min. Then, 3 ml of 10-3 M HAuCl4 were mixed with 18 ml of 2 mg/mL chitosan solution (medium molecular weight), and then heated to 50 °C. The colloidal solutions were centrifugated and re-suspended in ultrapure water. Loading of metformin hydrochloride (MET) (Sigma Aldrich) onto the surface of GNPc was performed by incubating the colloidal GNPc with MET at pH 3.5. The pH of the mixture was then adjusted to 8 with 1M NaOH. The obtained metformin gold nanoconjugates

(MET-GNPc) were subsequently washed to remove the free drug. All materials were purchased from Sigma Aldrich, unless otherwise specified.

Structural characterization of NPs – zeta potential, gold concentration, size

Optical extinction spectra of NPs were collected in a 2 mm quartz cell using a Jasco V-670 spectrophotometer with 1 nm spectral resolution. The zeta potential was recorded at 25 °C using a Malvern Zetasizer Nano ZS-90 instrument. The concentration of gold in the colloidal suspension (μg/mL) was determined by atomic absorption spectroscopy (Avanta PM, GBC-Australia). Transmission Electron Microscopy (TEM) was used in our previous study to determine the median size of GNPc (Aldea M, 2018).

Cell cultures

Our in vitro experiment used three glioblastoma cell lines and a normal endothelial cell line. GM1 is a primary glioblastoma cell line isolated from freshly resected glioblastoma specimens that has been shown to express both stem cell markers and neural markers, as previously described by our team (12). After isolation and expansion in a serum-free medium enriched with growth factors, GM1 cells were subsequently cultured in Ham's F-12 and DMEM media used in 1:1 ratio, supplemented with 15% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ ml streptomycin, 2mM L-glutamine, 1% non-essential amino acids (NEA), 55 μM beta-mercaptoethanol and 1 mM sodium pyruvate. Also, two commercial glioblastoma cell lines (A172 and U251, purchased from Sigma-Aldrich) were cultured in DMEM medium supplemented with 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ ml streptomycin, 1% NEA, 1 mM sodium pyruvate and 10% FCS.

Human endothelial cells (HUVEC), purchased from the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK) served as "normal cell model" and were cultured in RPMI medium, supplemented with 10% fetal calf serum (FCS), gentamicin 50 μg/ml, and amphotericin 100 μg/ml (Biochrom AG, Berlin, Germany).

Cultures were maintained at 37 °C in a humidified atmosphere of 95 % air and 5% CO2. All cell culture reagents were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA), unless otherwise specified.

NP administration and cell internalization

For each of the cell lines used, uncapped GNP, GNPc and MET-GNPc were administered at dose concentrations of 5, 10 and 20 μg/mL. Their internalization within cells was analyzed through dark field microscopy. Cells were cultured in Ibidi 30 μ-Dish of 50 mm and treated for 24 h at a concentration of 10 μg/mL of each nanoparticle. An inverted Zeiss Axio Observer Z1 microscope with a halogen lamp (HAL100, 100 W, Zeiss) focused on the sample at a constant intensity through a high numerical immersion dark field condenser (NA = 1.4) and the scattered light was collected by a LD Plan-Neofluar ×20 objective (NA = 0.4, Zeiss). Imaging acquisition was made with an AxioCam Icc digital camera and processed with the ZEN software.

Cell lines irradiation

In order to test if MET-GNPc could be used as a radiosensitizer, cell lines were irradiated at megavoltage energies of 1.25 MV with 2, 4 and 6 Gy, by using a Cobalt Theratron100R. Control experiments were performed following exactly the same treatment protocol, but without irradiation.

Cell viability after NP administration with or without irradiation

After reaching a sub-confluence of 60-80%, cells were incubated with 5, 10 and 20 μg/mL of each nanoparticle and free drug. After 24 h, cell viability was assessed via the MTT proliferation test, which measured the mitochondrial activity of living cells. After removing the medium and washing three times with PBS, the yellow MTT solution [3-(4,5-dimethylthiazolyl- 2)-2,5-diphenyltetrazolium bromide] was added and the plate was left at 37 ºC for 1 h to allow MTT to be metabolized. The resulting purple formazan was then re-suspended in 150 μl DMSO and placed on a shaking table. The reduction of MTT to formazan takes place only when mitochondrial enzymes are active; therefore, the conversion rate can directly estimate the number of living cells. The concentration was determined by optical density at 492 nm by using a fluorescence microplate reader (Synergy 2, BioTek, Winooski, VT, USA).

Statistical analysis

All experiments were repeated three times and expressed as mean ± SEM of three independent biological replicates. The comparison between groups was assessed by the one-way ANOVA followed by Bonfferoni's multiple comparison post-test. The statistical significance was set at $p < 0.05$ (GraphPad version 5.0 San Diego CA, USA).

Results

Physico-chemical characteristics of MET-GNPc and GNPc

The formation of the MET-GNPc nanoparticle was validated through spectroscopic measurements. By analysing the extinction spectra of MET-GNPc and GNPc, it may be observed that the coloidal solution maintaines its stability in the presence of MET as the form of the spectra does not change.

The optical spectra of colloidal solutions in Figure 1 exhibit a dominant extinction band located at 525 nm for GNPc, which shows the dipolar plasmon resonance of spherical NPs. For the MET-GNPc the plasmonic band has smaller wavelengths compared with GNPc. This difference is caused by the change of the refraction index of nanoparticles as a result of MET disposal near the gold surface after its incorporation within the polymeric matrix.

In the extinction spectrum of the MET-GNPc, a supplementary extinction band may be observed in the ultraviolet field at 230 nm, characteristic to MET, which proves the successful loading of GNPc with MET. MET loading efficiency and its quantity were assessed through UV-vis measurements of the initial MET used for incubation, of the supernatant collected after conjugation, of the mixture of MET-GNPc before and after centrifugation. As a supplementary control measurement, the concentration of loaded MET was measured by inducing an acid

medium (pH 2.5), which immediately led to the release of the entire MET quantity from the GNPc, followed by a 20 min centrifugation at 12.000 rotations per minute. MET concentration was determined by measurements of atomic absorption. In optimal incubation conditions, we observed that MET loads on GNPc at a concentration of 80 μM.

Also, the complex stability was monitored in time through UV-vis-NIR spectroscopy. It has been observed that in normal 4 degrees refrigerator conditions, the colloidal MET-GNPc maintains its stability for a minimum of 2 months.

In our previous work, we have already demonstrated a positive zeta potential and a medium size of nearly 26 nm of GNPc (Aldea M, 2018). The positive zeta potential appears as a result of the protonated amino groups from the chitosan chain which characterizes both GNPc and MET-GNPc.

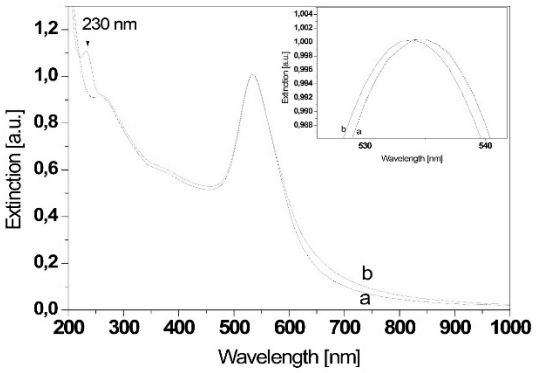

Figure 1 - UV-vis-NIR extinction spectra of GNPc (a) and MET-GNPc (b)

Cellular uptake of GNPs analyzed by dark field imaging

GNPs are easily visualized through dark field microscopy because of their surface plasmon resonances which make them able to strongly scatter the visible light. Cells were incubated at the same concentration of GNPc and MET-GNPc for 24h. The orange-red spots correspond to the light-scattering nanoparticles. As observed in Figure 2, both GNPc and MET-GNPc are highly internalized within the analyzed cell lines and they are located mostly throughout all the cytoplasm.

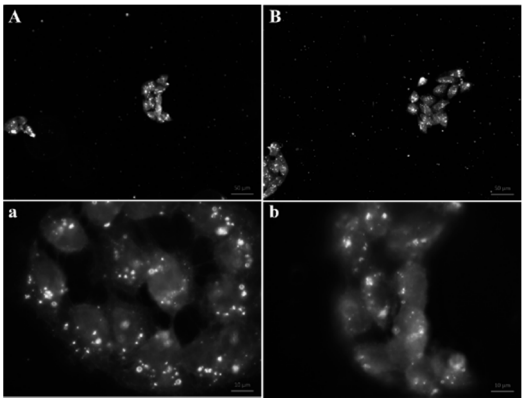

Figure 2 - Dark field microscopy images of GM1 cells incubated with GNPc and MET-GNPc for 24 h. (A), (a) GNPc; (B), (b) MET-GNPc

GNPc and MET-GNPc impacts the viability of glioblastoma cell lines

The tested cell lines included both glioblastoma stem-like cells (GM1) and commercial non-stem glioblastoma cells (A172 and U251) in order to investigate if the stem cell character confers any resistance or sensitivity to MET-GNPc. Also, an endothelial cell lines was used as a normal cell line.

The MTT viability test showed that the most sensitive cell lines were GM1 and U251, which were impacted by the 10 and 20 μg/mL concentrations of both GNPc and MET-GNPc ($p<0.001$) (Figure 3). Although MET-GNPc significantly affects cell proliferation when compared to control and uncapped GNPc, there is no difference when compared to the chitosan-capped GNPc ($p>0.05$). Therefore, it seems that the tumor cell viability is affected mainly by the chitosan component of the nanoparticles and that the benefit of MET loading is only minor and does not lead to an additional benefit in terms of cell viability.

MET-GNPc and GNPc slightly affect the endothelial cell line, especially when used at the 20 μg/mL concentration ($p<0.05$).

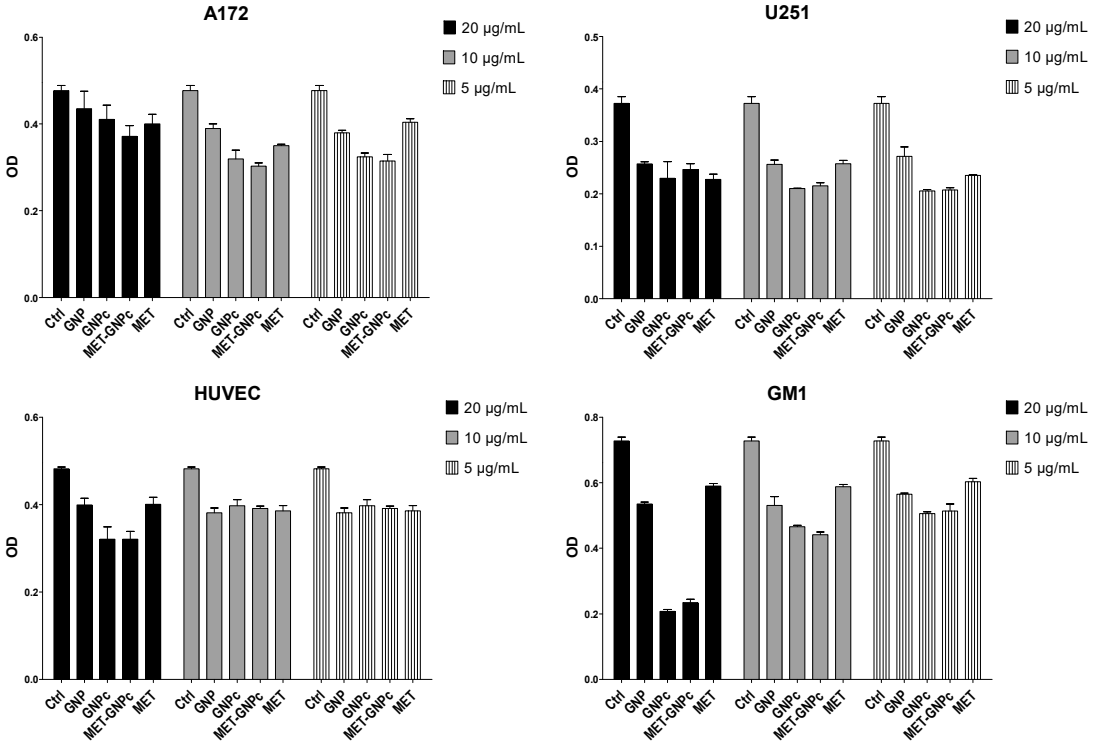

Figure 3 - MTT viability assay after 24h incubation with treatments (control, GNP, GNPc, MET-GNPc, MET). Increasing doses of each treatment (5 μg/mL, 10 μg/mL and 20 μg/mL) showed that cell viability was affected in a dose dependent manner (non-significant for the 5 μg/mL dose). Cell viability is mostly affected by the GNPc and MET-GNPc used at the 20 μg/mL concentration for each glioblastoma cell line ($p<0.001$). HUVEC cells were also affected by NP treatments, but only when treated with the 20 μg/mL concentration ($p<0.05$)

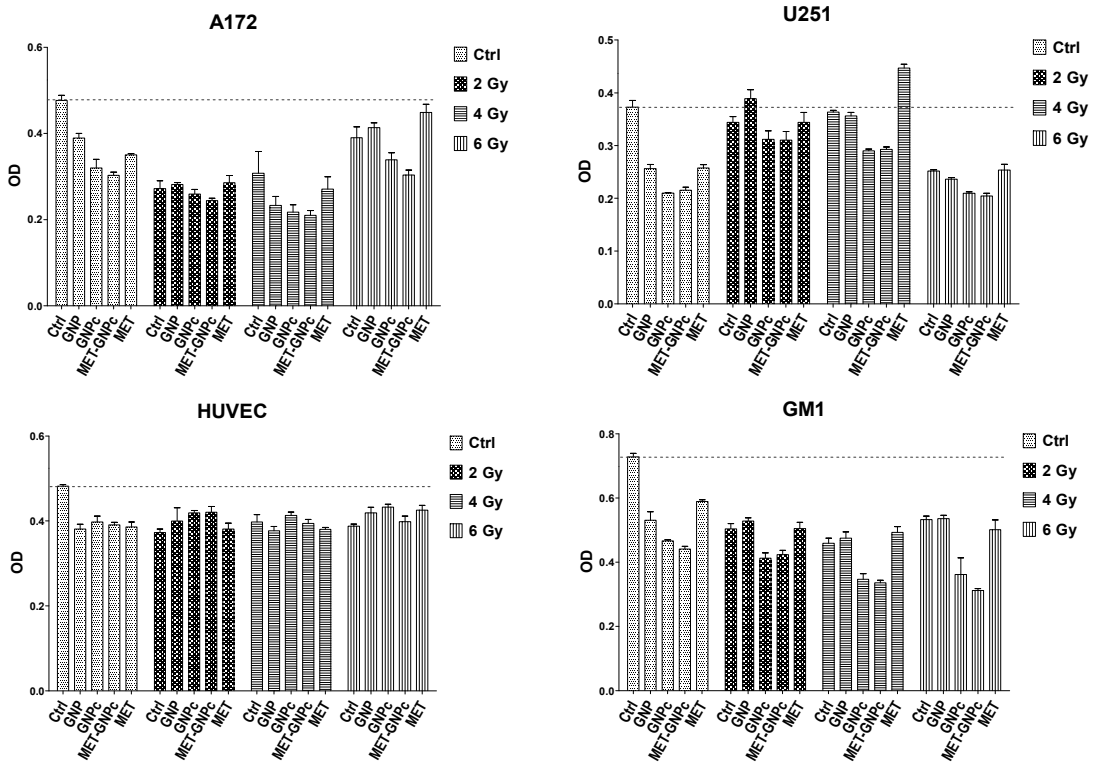

Figure 4 - Irradiated versus non-irradiated cells. Glioblastoma cell lines are affected by irradiation with a slight tendency of an enhanced response when combined to GNPc and MET-GNPc treatment ($p<0.05$). The addition of nanoparticles does not increase the toxicity of irradiation upon HUVEC, which are resistant to radiotherapy ($p>0.05$)

Radiotherapy shows no additional benefit when combined with GNPc or MET-GNPc

We investigated if such GNPc with or without MET could sensitize cells to irradiation. All tested NPs were used at a 10 μg/mL concentration and irradiated with increasing doses of 2, 4 and 6 Gy. HUVEC normal cell lines proved the highest resistance to irradiation and the combination between irradiation and nanoparticles did not impact their viability ($p>0.05$).

The response of the tumoral cell lines was different mainly depending on their proliferation index, but also on their intrinsic radioresistance. The A172 cell line was impacted by the 2 Gy and 4 Gy irradiation fractions, with a slight benefit when GNPc was combined with 4 Gy irradiation, but intriguingly, the 6 Gy dose was no different than the unirradiated control. The U251 cell line, which has a lower proliferation index, proved to be resistant at small doses of irradiation, but the combination of 4 Gy

irradiation and GNPc with/without MET was more toxic and decreased cell viability compared to each treatment alone ($p<0.05$). However, this additional benefit was not observed when the 6 Gy dose was used, despite the fact that this dose impacted cell proliferation.

The stem-like cell GM1 proved the best response when radiotherapy at higher doses was combined with nanoparticles as shown by their decreased viability when 4 and 6 Gy doses of irradiation were combined with MET-GNPc or GNPc (Figure 4).

Discussions

MET has shown an excellent anti-glioblastoma activity in numerous in vitro studies, by inhibiting the self-renewal capacity of glioblastoma stem cells, expression of stem cell markers, as well as their invasion and migration properties. However, the antiproliferative effects of MET were proven mainly when used at high drug concentrations, as many in vitro experiments used millimolar metformin concentrations (Song et al., 2018). From a translational point of view, such concentrations are not attained in the clinics by the maximal anti-diabetic dose normally used. Therefore, in this study, our aim was to test if an enhanced delivery and internalization of MET via nanoparticles, could overcome the limitation of small drug concentrations.

Nanotechnology holds an excellent potential of providing a preferential drug administration within the tumor, with an enhanced internalization and efficient drug delivery at the targeted site (Suri, Fenniri, & Singh, 2007). Our team previously synthetized chitosan-capped GNP and proved that such nanoparticles are highly internalized by glioblastoma-stem like cells and by normal osteoblasts cells. Intriguingly, GNPc proved a selective anti-proliferative effect for glioblastoma cells, as compared with un-capped GNP which had no anti-tumor effect. Despite an important intracellular accumulation within osteoblasts, GNPc showed no toxicity on the normal cell line. The increased intratumoral internalization of GNPc and their selective cytotoxicity on cancer cells have suggested that such NPs could be a promising vector for anti-cancer drugs (Aldea M, 2018).

Therefore, our team further synthetized GNPc and managed to functionalize them with small micromolar concentration of MET, in order to test if their enhanced delivery would lead to a therapeutic drug efficiency within the tumor cells. The synthesis of MET nanoparticles was an easy, reproducible method and resulted in the development of a stable compound, with physico-chemical characteristics similar to GNPc. However, although both GNPc and MET-GNPc had an important intracellular accumulation and proved to impact the survival of glioblastoma cells, there were no statistically significant differences between GNPc and MET-GNPc, suggesting that MET does not bring an additional benefit when used in small micromolar drug concentrations.

Considering the important anti-cancer effects proved by MET used in millimolar concentrations, further research must be made in order to find a suitable delivery method.

Kumar et al. synthetized a similar gold nanoparticle for the targeted delivery of metformin by capping GNPs with hyaluronic acid. The enhanced MET delivery was able to improve the anti-cancer activity of the MET-GNP complex compared to free MET in the treatment of liver cancer cells (Kumar, Raja, Sundar, Gover Antoniraj, & Ruckmani, 2015). An alternative way would be the development of NPs that would permit higher MET binding, by paying attention also to the fact that the increasing concentration of NPs would increase the quantity of excipients within the brain. Possible biodegradable NPs are more desirable for this purpose. Also, another strategy to increase drug concentrations would be the intratumoral administration of MET or within the tumoral cavity after surgical resection (Aldea et al., 2016).

Conclusion

Herein, we have developed a novel GNP loaded with MET, by using a biocompatible and biodegradable polymer, chitosan, as a reducing agent. This formulation exhibited high cell internalization due to the use of GNPc and had an increased anti-glioblastoma activity compared to free MET used in small micromolar concentrations. However, MET-GNPc does not add a significant benefit when compared to GNPc possible due to the small MET concentrations used for binding. Therefore, an increased internalization of MET-GNPc loaded with micromolar concentrations of MET does not prove the same anti-cancer effectiveness of millimolar concentrations of free MET. Improved delivery methods with an increased MET loading should be tested.

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