Self-assembling nanoparticles containing zoledronic acid: A new scenario for the treatment of glioblastoma

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ABSTRACT SUMMARY
Zoledronic acid (ZOL), a third generation bisphosphonate, is a breakthrough for the treatment of bone diseases1. Interestingly, several studies have confirmed a potent anti-cancer activity of ZOL in vitro. Unfortunately, only a limited and not effective activity of ZOL has been shown in vivo. This discrepancy has been attributed to the short plasma half-life of ZOL and to its rapid accumulation within bone1. To address this problem, new self-assembling PEGylated nanoparticles (PLCaPZ NPs) have been previously developed in our lab to use ZOL for the treatment of tumors2,3. Here, in order to overcome the blood brain barrier (BBB) and to actively target specific cancer cells for the treatment of brain tumors, PLCaPZ NPs were functionalized with human Transferrin (Tf). Tf-modified NPs (Tf-PLCaPZ NPs) were designed with a self-assembling strategy to be prepared before use. The in vitro activity of Tf-PLCaPZ NPs was evaluated in LN-229, U-373 MG, U-87 MG, glioblastoma cell lines. In all the cell lines, Tf-PLCaPZ NPs strengthened the growth inhibition induced by free ZOL. In vivo, in nude mice xenografted with glioblastoma U373-MG cells, the treatment with Tf-PLCaPZ NPs elicited the highest anti-cancer activity compared to all the other treatments.

INTRODUCTION
ZOL is the most potent inhibitor of the bone resorption, largely used in clinic for the treatment of bone diseases (i.e. bone metastases, osteoporosis and Paget's disease). Numerous in vitro studies have demonstrated a powerful pro-apoptotic effect of ZOL in several tumor cell lines, suggesting the use of ZOL as anticancer drug1. However, the short plasma half-life and the rapid accumulation of ZOL in bone, hamper the achievement of effective drug levels in tumors located outside the skeletal compartment. Previously, self-assembling NPs containing ZOL were developed in our lab2. These NPs strongly increased the in vivo anticancer activity of ZOL. The purpose of this study was the development of Tf-modified self-assembling NPs to deliver ZOL in brain tumors, such as glioblastoma.

EXPERIMENTAL METHODS
PEGylated self-assembling NPs were prepared as follows. Briefly, two aqueous solutions of CaCl2 and Na2HPO4, respectively, were mixed under magnetic stirring and the resulting dispersion was filtered through 0.22 μm pore filters (CaP NPs). The dispersion was then mixed with an aqueous solution of ZOL, resulting in Ca2+/PO43- NPs complexed to ZOL (CaP NPs). In the following step, CaP NPs were mixed with PEGylated cationic liposomes for 10 min at room temperature to obtain PLCaPZ NPs. To prepare Tf-modified NPs, PEGylated cationic liposomes were incubated with an aqueous solution of Tf. The resulting Tf-PEGylated cationic liposome complex was allowed to stand at room temperature for 15 min. In the following step, CaP NPs were mixed with Tf-PEGylated cationic liposomes complex for 10 min at room temperature, obtaining Tf-PLCaPZ NPs. The NPs were characterized in terms of mean diameters and size distribution, zeta potential (ζ), morphology and ZOL complexation efficiency. The in vitro activity and cytotoxicity of Tf-PLCaPZ NPs were evaluated in LN-229, U-373 MG, U-87 MG, glioblastoma cell lines. Cellular proliferation studies in the presence of ZOL or ZOL containing formulations were carried out by MTT assay. Finally, the antitumor activity of Tf-PLCaPZ NP was evaluated in an orthotropic model of glioblastoma, U373-MG-LUC xenografts.

RESULTS AND DISCUSSION
The characteristics of the formulations containing ZOL are reported in Table 1. PLCaPZ NPs, with and without Tf, had a mean diameter of about 150 nm with PI< 0.2. ζ of PLCaPZ NPs was significantly reduced, when Tf was added to the preparation (Table 1).

Table 1. Physical characteristics of the formulations developed.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Diameter (nm) ± SD</th>
<th>ζ ± SD</th>
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<tbody>
<tr>
<td>PLCaPZ NPs</td>
<td>147.5 ± 7.1</td>
<td>+17.5 ± 5.6</td>
</tr>
<tr>
<td>PEGylated cationic liposomes</td>
<td>140.0 ± 15.0</td>
<td>+14.9 ± 4.0</td>
</tr>
<tr>
<td>Tf-PEGylated cationic liposomes complex</td>
<td>142.4 ± 21.1</td>
<td>+7.5 ± 2.0</td>
</tr>
<tr>
<td>Tf-PLCaPZ NPs</td>
<td>147.7 ± 15.0</td>
<td>+11.3 ± 1.1</td>
</tr>
</tbody>
</table>

HPLC analysis of non-complexed ZOL showed an actual loading of 100 μg of ZOL/mg lipids, corresponding to a complexation efficiency of about 100%.
In all the tested cell lines, the Tf-PLCaPZ NPs strengthened the growth inhibition induced by free ZOL (Figure 1). However, if compared with PLCaPZ NPs, the cytotoxicity of TF-PLCaPZ NPs was increased only in the case of LN-229 (glioblastoma cells with over-expressed levels of Tf-receptors) (Figure 1). In the other cell lines, the incubation with TF-PLCaPZ NPs vs PLCaPZ NPs,
resulted in a reduced inhibition of cell growth (data not showed).

![Graph](image)

*P<0.05 vs respective control
** P<0.01 vs respective control
§ P<0.05 vs respective zoledronate
ǂ P<0.01 vs respective zoledronate

Figure 1. Cytotoxicity of PLCaPZ NPs and TF-PLCaPZ NPs on LN-229 glioblastoma cell lines.

In *in vivo* studies, a significant reduction in tumor growth and an increased survival following administration of TF-PLCaPZ NPs was observed in an intracranial model of glioblastoma (Figure 2). It is worthy of note that, following treatment with TF-PLCaPZ NPs, almost a complete remission of the tumor mass, only after 23 days of treatment, in a significant percentage of animals, was observed (Figure 2).

![Images](image)

Figure 2. Representative images of luminescence associated to injected tumor cells in untreated mice (Control), in mice treated with blank TF-PLCaPZ NPs (Blank TRF-NPs), and in mice treated with TF-PLCaPZ NPs (TRF-NPs-ZOL). U373-MG LUC cells were injected intracranially through the center-middle area of the frontal bone in nude mice. After 7 days mice were randomized and treated with Plain TF-PLCaP NPs (8 mice) and TF-PLCaP NPs (8 mice) at 20 μg mouse i.v. For three times a week for three consecutive weeks. Tumor growth of xenografts was monitored using IVIS imagin system.

Finally, the intracranial distribution of TF-PLCaPZ NPs was evaluated in frozen sections after the different treatments. In Figure 3 is shown the intracranial distribution of double labeled FITC/TRITC TF-PLCaPZ NPs in untreated group (*left*) and in TF-PLCaPZ NPs treated group (*right*). Only after 6 hours of double labeled TF-PLCaPZ NPs injection, a consistent accumulation of TF-PLCaPZ NPs (yellow signal) in the tumor mass (T) was already evident (Figure 3, *left*, star). Interestingly, after 3 weeks of treatment with TF-PLCaPZ NPs a strong reduction of the tumor mass was observed (Figure 3, *right*). Moreover, TF-PLCaPZ NPs were widely distributed in the tumor mass and in some brain cortex cells (Bc).

![Images](image)

Figure 3. Distribution of TF-PLCaPZ NPs in brain samples Two weeks after tumors injection, the mice were treated with FITC/ TRITC- double labeled TF-PLCaPZ NPs, given i.v. at 20 μg mouse. Frozen brain sections were examined by fluorescence microscope after nuclear DAPI counterstain. *Left*, Tumor (T) developed in the basal brain nuclei (Bbn) from a mouse sacrificed 6 hours after the first injection of FITC/TRITC- TF-PLCaPZ NPs. *Right*, Tumor (T) developed in the brain cortex (Bc) from a mouse treated with FITC/ TRITC-NPs-ZOL given three times a week for 3 consecutive weeks. Original magnification, 100 X. The insert shows the FITC/ TRITC-NPs-ZOL uptake in the cytoplasm of tumor cells. Original magnification, 400 X.

**CONCLUSIONS**

New Tf-modified self-assembling NPs containing ZOL were developed in our lab. A potent anti-tumor effect of ZOL, after the use of TF-PLCaPZ NPs, has been demonstrated in an animal model of glioblastoma. These findings open a new scenario in the treatment of brain tumors, for which a paucity of effective treatments exists.

**REFERENCES**

