

TITLE

Anti-cancer efficacy of intravenously administered tumor-targeted vesicles entrapping tocotrienol

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RUNNING TITLE

Tumor-targeted tocotrienol for cancer therapy

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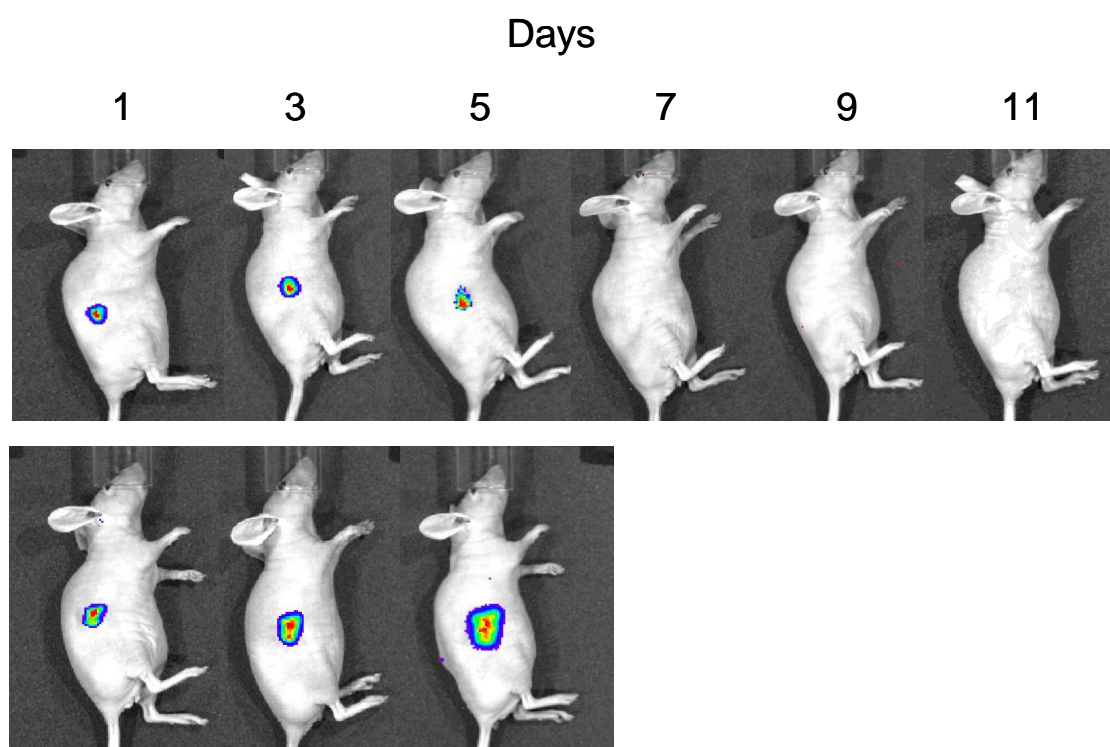
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ABSTRACT

Despite its potent *in vitro* anti-cancer activity, the vitamin E extract tocotrienol has its therapeutic potential hampered by its poor bioavailability and by its inability to reach tumors in a specific way after intravenous administration. One possibility to overcome this issue would be to entrap tocotrienol within vesicles bearing transferrin, whose receptors are present in abundance on many cancer cell types. In this study, we demonstrated that the systemic administration of tocotrienol entrapped within transferrin-bearing vesicles led to tumor suppression of 20% of A431 epidermoid carcinoma tumors and 50% of B16-F10 melanoma tumors at the end of the treatment. The survival of animals treated with these vesicles was improved by more than 20 days in comparison with the controls, for the two cancer models tested. Animals did not show any secondary effects following administration of the treatment. The entrapment of tocotrienol within transferrin-bearing vesicles is therefore a promising therapeutic strategy, which could result in tumor suppression after systemic administration of this delivery system.

GRAPHICAL ABSTRACT

Bioluminescence imaging of the tumor regression resulting from the intravenous administration of transferrin-bearing vesicles entrapping tocotrienol, in a mouse bearing subcutaneous B16-F10-luc tumors. (Controls: untreated tumors).



KEYWORDS

Cancer therapy; delivery system; *in vivo*; tocotrienol; transferrin; tumor targeting

INTRODUCTION

Tocotrienol, a compound of the vitamin E family, has recently gained considerable attention due to its potent anti-cancer activity displayed *in vitro* on many types of cancer, including prostate, breast, pancreatic, colorectal, lung and liver cancer [1- 4]. It has also been used as a therapeutic adjuvant, leading to synergistic anti-proliferative effects when associated with various anti-cancer drugs such as tamoxifen, celecoxib and gemcitabine [5-9]. However, its therapeutic use against cancer *in vivo* has been prevented by its poor bioavailability and its inability to specifically reach tumors. In order to overcome these problems, we recently entrapped the tocotrienol-rich fraction (TRF) of palm oil in tumor-targeted vesicles bearing transferrin [10]. Transferrin (Tf) is a particularly interesting ligand for active tumor targeting, as its receptors are expressed in abundance on many types of cancers [11]. Transferrin-bearing vesicles provides a tumor-selective targeting strategy in addition to its passive targeting, resulting from the enhanced permeability and retention of particulate delivery systems in tumors [12]. Transferrin has been widely used as a tumor-targeting agent for tumor-targeted drug and gene delivery [11, 13-17]. We recently demonstrated that the intravenous injection of transferrin-conjugated vesicles entrapping TRF resulted in regression of the tumors shortly after administration of the treatment. However, this therapeutic effect was short-lived and only lasted for the duration of the treatment. We now seek to increase this therapeutic effect by optimizing the frequency of administration of the TRF vesicle formulation. The objectives of this work are therefore to assess the *in vivo* therapeutic efficacy of this optimized treatment.

MATERIALS AND METHODS

Materials

Tocotrienol Rich Fraction (TRF) of palm oil corresponds to a mixture of various tocotrienol isomers (17.6% α -tocotrienol, 15.1% δ -tocotrienol and 23.1% γ -tocotrienol), 15.3% α -tocopherol, and other tocotrienol-related compounds. TRF was a kind gift from Dr. Abdul Gapor (Malaysian Palm Oil Board, Kuala Lumpur, Malaysia).

Solulan C24 came from Amerchol (Edison, NJ). A431 human epidermoid carcinoma and Bioware[®] B16-F10-luc-G5 mouse melanoma expressing the firefly luciferase, were respectively obtained from the European Collection of Cell Cultures and from Caliper Life Sciences (Hopkinton, MA). Media needed for the culture of these cells was purchased from Invitrogen (Paisley, UK). All other reagents and chemicals that are not specifically mentioned below were purchased from Sigma Aldrich (Poole, UK).

Preparation of transferrin-bearing vesicles entrapping TRF

Transferrin-conjugated vesicles entrapping TRF were prepared and characterized as previously described [10]. To prepare control vesicles entrapping TRF, a mixture of sorbitan monostearate (Span 60; 65 mg), cholesterol (58 mg), Solulan C24 (54 mg) in 2 mL TRF solution (0.5 mg/mL, prepared in dimethylsulfoxide) was shaken at 60°C for 1 hour, before being probe sonicated with a Soniprep 150 (MSE, United Kingdom) for 4 min. Tf-bearing vesicles were then prepared by conjugating Tf (6 mg) to the control vesicles (2 mL), using dimethylsuberimidate as a cross-linker [10].

Untrapped TRF, dimethylsulfoxide, free Tf and dimethylsuberimidate were removed by ultracentrifugation (150 000 g for 1h). The drug loaded vesicle pellet was then resuspended in 2 ml PBS.

TRF loading in the vesicles was measured by spectrofluorimetry (λ_{ex} 295nm, λ_{em} 325nm) using a Varian Cary eclipse fluorescence spectrophotometer (Agilent Technologies, CA) after disruption of the vesicles with isopropanol.

Cell culture

A431 and B16-F10-luc-G5 cancer cell lines overexpressing transferrin receptors were respectively cultivated in DMEM or RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin, in a humidified atmosphere of 5% CO₂ at 37°C.

***In vivo* tumoricidal activity**

The *in vivo* experiments in this study were approved by the local ethics committee and were conducted according to the UK Home Office regulations.

A431 and B16-F10-luc-G5 cells were subcutaneously injected to both flanks of female immunodeficient BALB/c mice (1×10^6 cells per flank). Vascularized tumors, with a typical diameter of 5 mm, were palpable 6 days after implantation of the cells. The mice were then intravenously injected via the tail vein with TRF entrapped in Tf-bearing vesicles, control vesicles or in solution (10 μg TRF per injection for all treatments, 5 mice per treatment). The administration of the treatment was done once daily for 20 days.

The weight of the animals was measured daily as a surrogate marker of toxicity. The volume of the tumors was also determined daily, by caliper measurements (volume = $d^3 \times \pi/6$). At the end of the experiment, the tumor response to treatment was classified according to the Response Evaluation Criteria in Solid Tumors [18]: progressive disease, when the relative tumor volume has increased by at least 1.2-fold compared to its volume at the start of the experiment, stable disease when the relative tumor volume is comprised between 0.7 and 1.2-

fold compared to its starting volume, partial response when the relative tumor volume is smaller or equal to 0.7-fold, and complete response when the tumor has completely disappeared.

The therapeutic efficacy of the treatments was also assessed by bioluminescence imaging, using an IVIS Spectrum (Caliper Life Sciences, MA). Mice bearing subcutaneous B16-F10-luc-G5 tumors were intravenously injected with the treatments described above. Ten minutes before imaging, they were intraperitoneally injected with D-luciferin, the substrate of luciferase (150 mg D-luciferin /kg body weight) and anaesthetized by isoflurane inhalation on Days 1, 3, 5, 7, 9 and 11 of the experiment. D-luciferin-expressing bioluminescent tumors were able to emit some light, which was detected for a constant duration of 2 min using Living Image[®] software. All images were acquired using the same illumination settings.

Statistical Analysis

Results were expressed as means \pm standard error of the mean (S.E.M). Statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey multiple comparison post-test (Minitab[®] software, State College, PE). Differences were considered statistically significant for P values lower than 0.05.

RESULTS

Intravenously administered TRF entrapped in Tf-bearing vesicles led to tumor regression within 72 hours on A431 tumors (Fig. **1A**). This effect was maintained for 15 days, allowing the majority of the tumors to reach a size about half their initial size. From Day 15, while some tumors kept regressing, others started growing, which led to an overall slowdown of A431 tumor growth in comparison to the tumor growth observed following administration of the other treatments. From Day 24, the mice bearing growing tumors reaching the maximum allowed size (13 mm) had to be euthanized. The other mice, with tumors smaller than 13 mm diameter, were kept in the study. By contrast, none of the other treatments resulted in any A431 tumor regression. The effects on tumor growth observed following treatment with Tf-bearing vesicles entrapping TRF were significantly different from those resulting from the other treatments.

The replacement of A431 tumors by B16-F10-Luc tumors resulted in a different pattern of tumor growth. The treatment with TRF entrapped in Tf-bearing vesicles led to the decrease of all tumors for the 3 first days of the experiment (Fig. **1B**). From Day 3, although some tumors kept regressing, others started growing, which led to an overall B16-F10 tumor growth similar to that observed following treatment with control vesicles until Day 7. From Day 7, the mice bearing tumors that reached the maximum allowed size had to be euthanized. The overall tumor size was decreasing from Day 7 to Day 25, until two of the tumors which were regressing started growing again, resulting in the sacrifice of the mouse carrying them at Day 28. The experiment was prolonged by 3 days compared to the A431 tumor growth experiment to assess if any other tumors would start growing again, which was not the case. For this cell line as well, none of the other treatments resulted in tumor regression. The effects on tumor growth observed following treatment with Tf-bearing vesicles entrapping TRF were

significantly different from those resulting from the other treatments from Day 8 to Day 33 (end of the experiment).

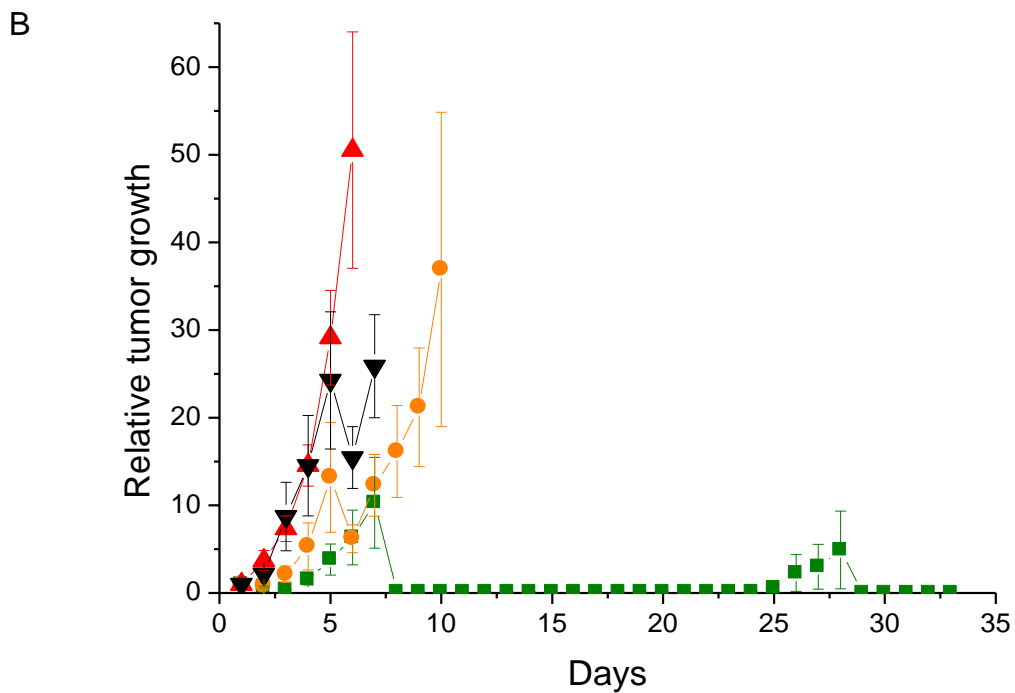
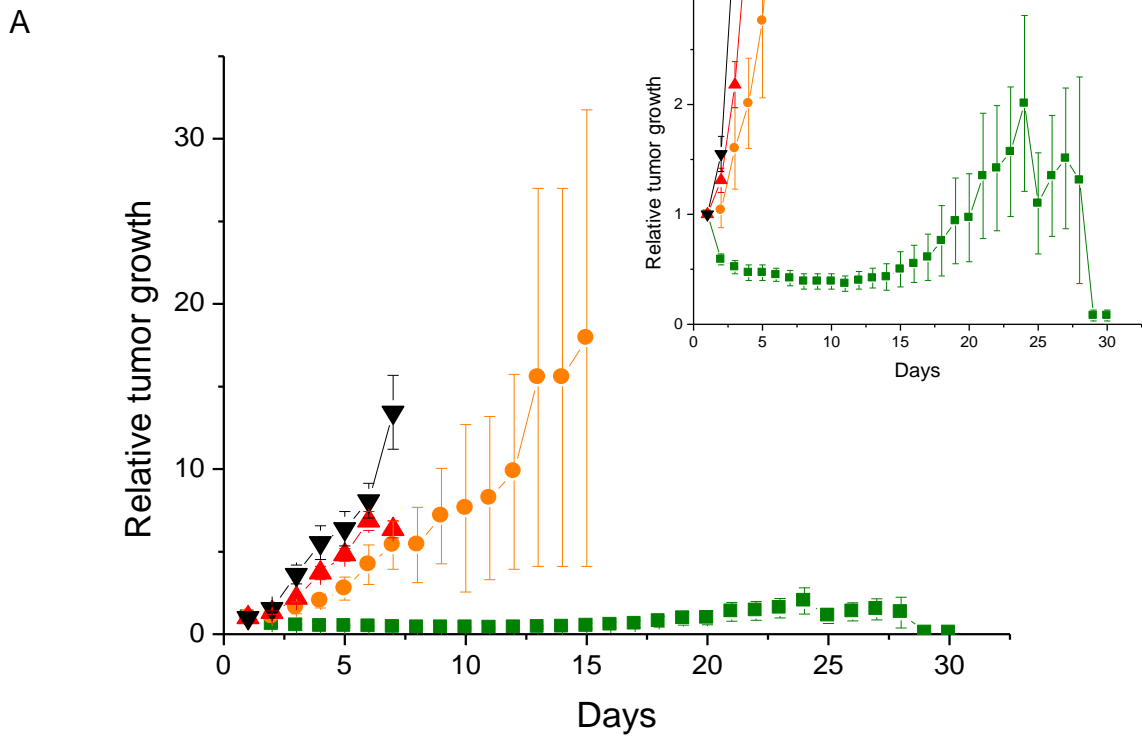


Fig. (1). Tumor growth studies following systemic administration of Tf-bearing vesicles entrapping TRF (10 $\mu\text{g}/\text{injection}$) (green) in a mouse bearing A431 tumors (A) or B16-F10-Luc tumors (B). Controls: control vesicles (without Tf) entrapping TRF (orange), TRF solution (red), untreated tumors (black) (inset: magnification of Figure 1A) (n=10).

Bioluminescence imaging qualitatively confirmed the tumoricidal activity of TRF entrapped in Tf-vesicles (Fig. 2). B16-F10-luc tumors treated with Tf-vesicles entrapping TRF regressed at Day 5, before completely disappearing from Day 7. On the other hand, all the other treatments resulted in tumor growth.

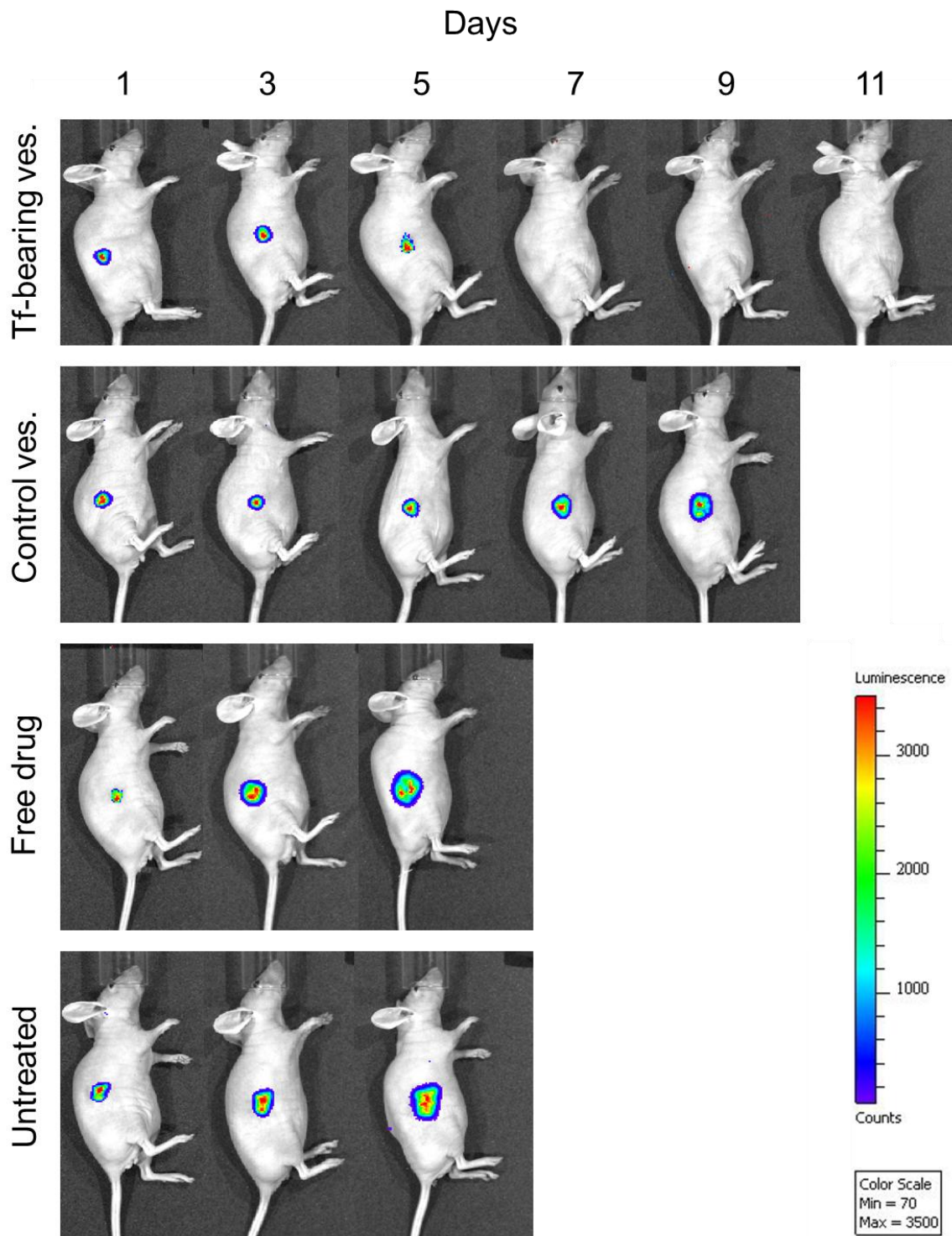


Fig. (2). Bioluminescence imaging of the tumoricidal activity of TRF entrapped in Tf-bearing vesicles in mice bearing subcutaneous B16-F10-luc tumors. Controls: TRF administered as entrapped in control vesicles or in solution, untreated tumors. The scale indicates surface radiance (photons/s/cm²/steradian).

The administration of TRF entrapped in vesicles or in solution was well tolerated by the mice and no visible signs of toxicity or significant weight loss were observed during the study (Fig. 3).

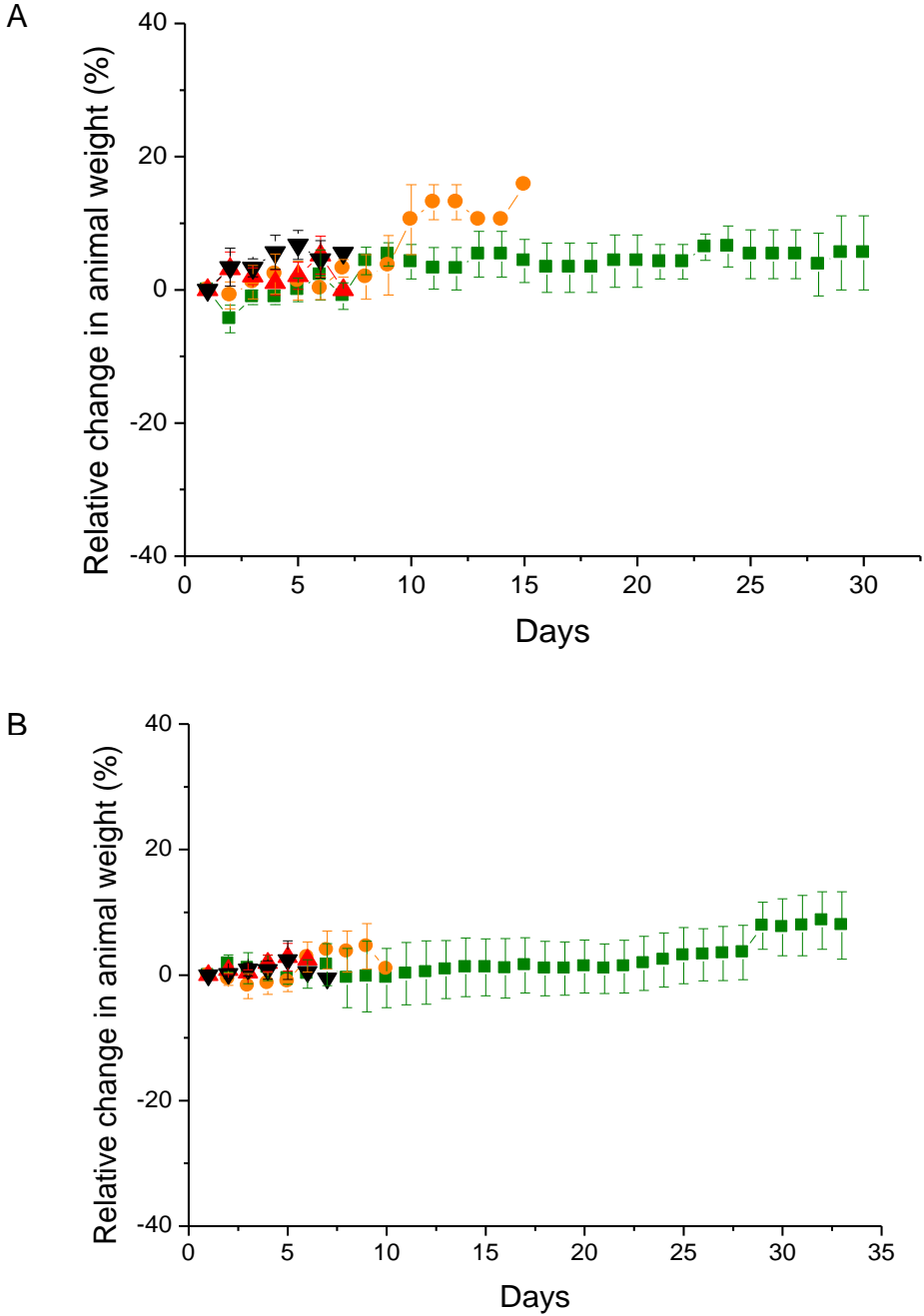


Fig. (3). Variations of the animal body weight following systemic administration of Tf-bearing vesicles entrapping TRF (10 $\mu\text{g}/\text{injection}$) (green) in a mouse bearing A431 tumors (A) or B16-F10-Luc tumors (B). Controls: TRF administered as entrapped in control vesicles (orange) or in solution (red), untreated tumors (black) (n=10).

On the last day of the experiment, treatment with Tf-bearing vesicles entrapping TRF resulted in complete tumor disappearance for 20% of A431 tumors and tumor regression for another 20% of A431 tumors (Fig. **4A**). Treatment with control vesicles also led to tumor regression, but with the less positive outcome of 10% partial response and 10% stable response. On the other hand, all the tumors treated with TRF solution or left untreated were growing.

The tumor response to treatment was more successful for B16-F10 tumors. On the last day of the experiment, 50% of B16-F10 tumors treated with Tf-bearing vesicles entrapping TRF had completely disappeared, while another 10% of tumors had regressed (Fig. **4B**). By contrast, all the other treatments resulted in 100% tumor growth.

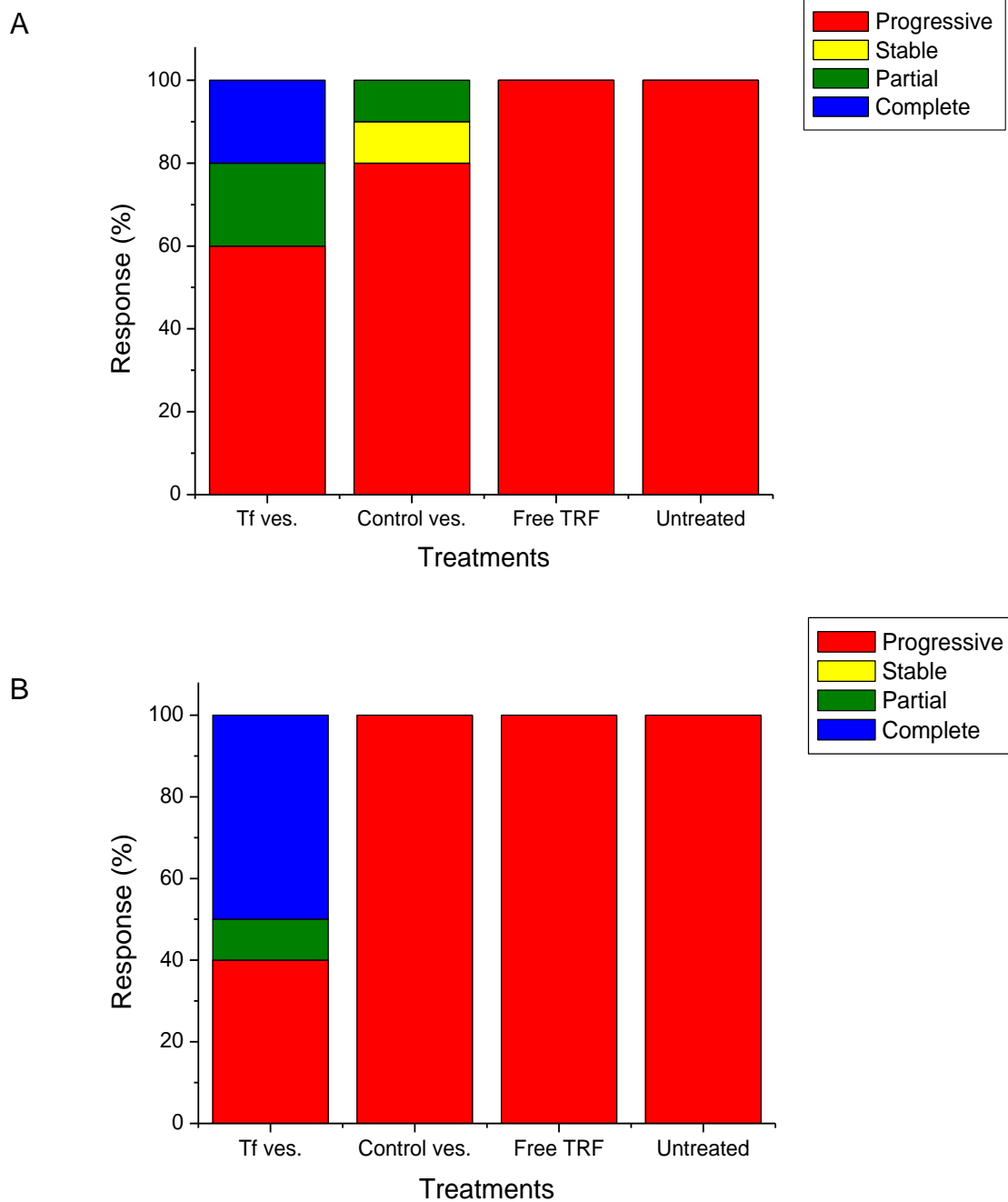


Fig. (4). Overall tumor response to treatments following systemic administration of Tf-bearing vesicles entrapping TRF (10 $\mu\text{g}/\text{injection}$) in a mouse bearing A431 tumors (A) or B16-F10-Luc tumors (B). Controls: TRF entrapped in control vesicles or in solution, untreated tumors (n=10).

As a result of this improved therapeutic effect, the survival of A431 tumors-bearing mice treated with Tf-bearing and control vesicles was extended by 23 days and 8 days respectively, in comparison with the mice receiving no treatment (Fig. **5A**). The survival was even improved on B16-F10 tumors-bearing mice, as it was respectively extended by 26 days and 3 days after treatment with Tf-bearing and control vesicles, compared to untreated mice (Fig. **5B**). However, the administration of TRF solution did not extend the survival of the animals compared to untreated mice with both tumor cell lines.

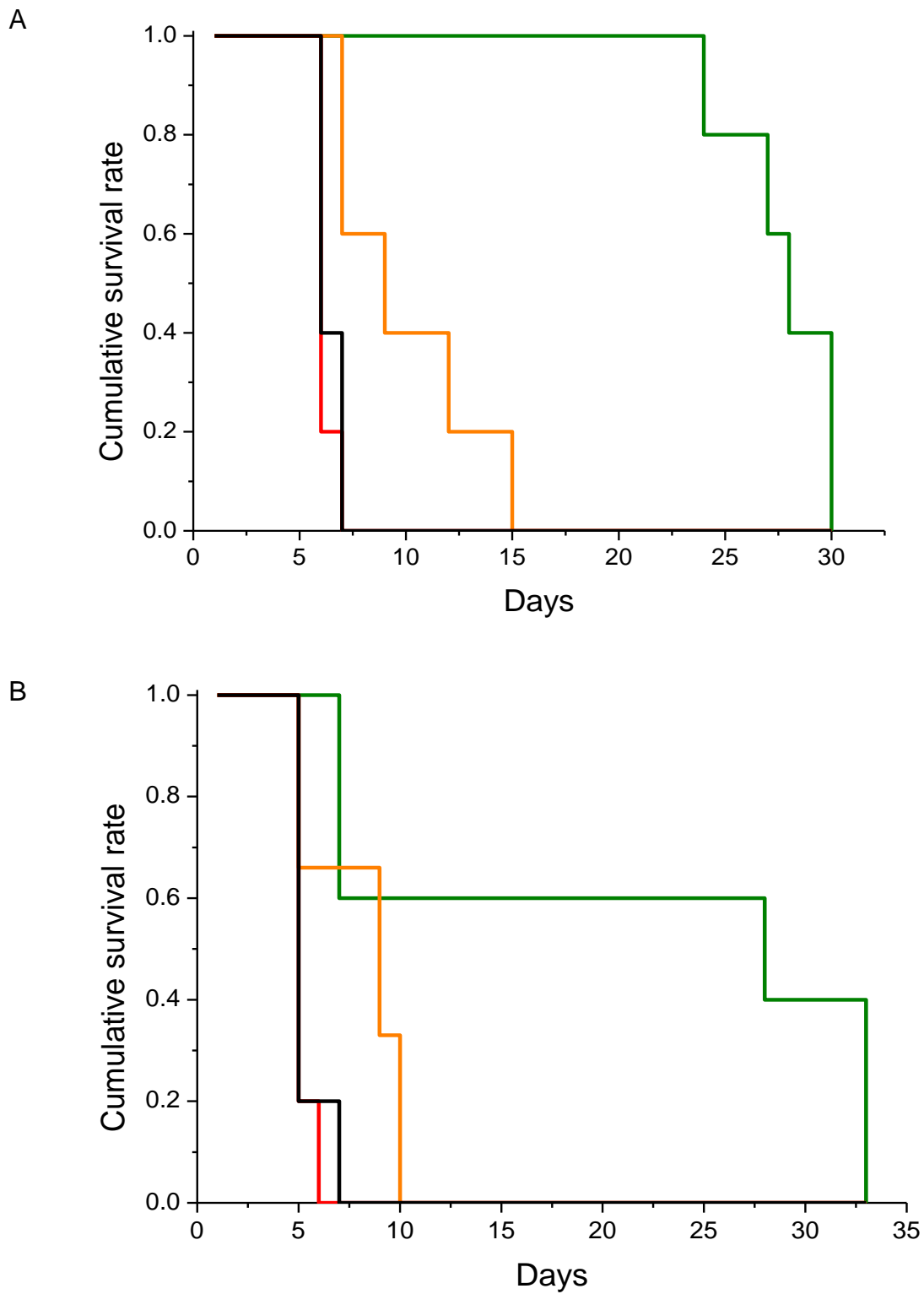


Fig. (5). Cumulative survival rate of mice bearing A431 tumors (A) or B16-F10-Luc tumors (B) following systemic administration of Tf-bearing vesicles entrapping TRF (10 $\mu\text{g}/\text{injection}$) (green). Controls: TRF entrapped in control vesicles (orange) or in solution (red), untreated tumors (black) (n=10).

DISCUSSION

Although highly promising *in vitro*, the use of the tocotrienol for the treatment of cancer has mainly been hampered by the inability of this drug to specifically reach tumors following systemic injection. To overcome this issue, we hypothesized that entrapping TRF in drug delivery systems bearing transferrin, whose receptors are present in abundance on cancer cells, would increase the amount of drug delivered to cancer cells and therefore increase its therapeutic efficacy.

In this study, the intravenous injection of tocotrienol entrapped in transferrin-bearing vesicles led to a complete tumor disappearance of 20% of A431 tumors and 50% of B16-F10 tumors. Consequently, tumor-bearing mice treated with this formulation showed an extended survival of more than 20 days compared to untreated mice.

This work corresponds to a major improvement of the therapeutic efficacy of this drug for the treatment of tumors. Tocotrienol co-encapsulated with simvastatin in lipid nanoparticles had already been shown to have an improved anti-proliferative effect against +SA mammary epithelial cancer cell line, in comparison with control α -tocopherol nanoparticles (with respective IC_{50} of 0.52 μ M and 17.7 μ M) in *in vitro* experiments. However, no *in vivo* results have been reported with this delivery system so far [19]. In our previous studies with the Solulan-based vesicles, we demonstrated that the systemic administration of TRF entrapped in Tf-bearing vesicles were able to decrease the size of A431 tumors, but only for the duration of the treatment, which was 10 days [10]. All the tumors then grew back after halting the treatment. None of them disappeared at any time when treated with only 10 injections. By contrast, our current regimen of administration of 20 injections increased the therapeutic effect on both tested tumors, resulting in complete disappearance of 20% and 50% of them, respectively. In addition, this therapeutic effect was maintained after halting the treatment. The results obtained in this current study were even improved compared with those obtained

with our intravenously administered Tf-conjugated, tocopheryl-based multilamellar vesicles entrapping tocotrienol, which resulted in tumor eradication of 20% of A431 tumors and 40% of B16-F10 tumors [16]. These results demonstrate that tocotrienol is able to exert an anti-cancer therapeutic effect *in vivo*, not only as a tumor growth inhibitor or as a therapeutic adjuvant as previously described [1, 20], provided it is targeted to the tumors. In addition, this targeted nanomedicine was able to reach subcutaneous tumor models overexpressing transferrin receptors, following intravenous injection and should therefore have the potential to reach metastatic tumors disseminated in the body.

The mechanism of action used by tocotrienol to exert its therapeutic effect in our study is still unknown. Tocotrienol has been shown to be able to activate p53, induce apoptosis and modulate Bax/Bcl-2 ratio [21]. In addition, it can inhibit angiogenesis by down regulating the expression of the vascular endothelial growth factor receptor [22]. It also exerts its anti-cancer effect by inhibiting DNA polymerase and telomerase, enzymes involved in the proliferation of cancer cells [23-24]. The extent of the therapeutic effect observed in our study made us suggest that many of these mechanisms were involved in the anti-cancer effect observed. There may be scope for further improvements in the *in vivo* activity of these nanomedicines by understanding the mechanisms behind the variability of the response between individual tumors, hopefully resulting in a further optimized therapeutic effect against tumors.

These therapeutic effects and the good tolerability of the treatments potentially make the transferrin-conjugated vesicles entrapping tocotrienol a highly promising nanomedicine, which will be further investigated.

CONCLUSION

The entrapment of tocotrienol in transferrin-conjugated vesicles significantly increased the therapeutic efficacy of tocotrienol *in vivo* compared to the drug solution. The systemic injection of tocotrienol entrapped in these targeted vesicles resulted in the disappearance of 20% of A431 tumors and 50% of B16-F10 tumors on the last day of the experiment. Consequently, the survival of the tumor-bearing mice was extended by more than 20 days compared to untreated mice, on both cancer cell lines. Transferrin-vesicles entrapping tocotrienol is therefore a highly promising therapeutic system which deserves further investigation.

CONFLICT OF INTEREST

The authors have no competing interests.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

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