Growth inhibition of human ovarian carcinoma by a novel AvidinOXanchored biotinylated camptothecin derivative

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A B S T R A C T

Oxidized form of avidin, named AvidinOX, provides stable fixation of biotinylated molecules in tissues, thus representing a breakthrough in topical treatments. AvidinOX proved to be a stable receptor for radiolabeled biotin, biotinylated antibodies and biotinylated cells. In order to expand applicability of the AvidinOX-based delivery platform, in the present study we investigated the possibility to hold biotinylated chemotherapeutics in AvidinOX-treated sites. A novel biotinylated Gimatecan-derived camptothecin coded ST8161, was injected at suboptimal doses into human tumors xenografted in mice, alone or pre-complexed to AvidinOX. Significantly higher tumor growth inhibition was observed when the drug was complexed to AvidinOX , suggesting the potential utility of this delivery modality for the local treatment of inoperable tumors.

Keywords: AvidinOX, tumor xenograft , camptothecin, biotin, Gimatecan

The choice of anti-cancer therapy depends on the cancer's type, location and stage of the disease. Methods of experimental cancer treatment are continuously under development. For localized inoperable cervical, prostate, breast, skin and many other cancers and, even for liver and pulmonary oligometastasis, brachytherapy has been recognized as an effective form of local irradiation[1-5]. Brachytherapy can be performed by applying radiation sources within or close to the tumor by using devices charged with radioisotopes and the technology is meant to minimize the radioactive exposure of non-target organs. Brachytherapy devices include balloons, catheters and permanent seeds requiring highly skilled medical personal and sophisticated after loading equipment to avoid radiation exposure of clinical staff[6]. Safety issues have been raised with brachytherapy procedures. Particularly, the injection of radioactive seeds has been reported to induce in some cases long-term complications due to seed migration to the brain[7], heart[8] or lung where they provoke embolization of pulmonary arteries[9].

We recently reported that the oxidized form of avidin named AvidinOX, exhibits the distinctive property to form Schiff's bases with tissue proteins while maintaining the capacity of original avidin to bind biotin and biotin derivatives with high affinity[10-13]. AvidinOX provides stable fixation of radiolabeled biotin in tissues thus representing a new biological form of brachytherapy. This product is currently under investigation in phase I clinical trials for targeting ^{177}Lutetium-biotinDOTA (^{177}Lu-ST2210) to inoperable tumor lesions and liver metastases (ClinicalTrials.gov NCT02053324 and NCT03188328). Previous data from our group showed that AvidinOX can be employed for targeting diverse biotinylated therapeutics including cells[14] and antibodies.[15-17] Our studies indicated that AvidinOX-anchored antibodies including biotinylated Cetuximab (bCet) and Panitumumab

(bPan) or Trastuzumab and Pertuzumab exhibit much higher inhibitory activity against tumor cells as compared to their original non-biotinylated versions.

In order to expand applicability of the AvidinOX-based delivery platform, in the present paper we investigated the possibility to hold biotinylated chemotherapeutics in AvidinOX-treated sites. Therefore, ST8161, a novel biotinylated camptothecin-derived agent, was complexed to AvidinOX and injected directly into the tumor in comparison with ST8161 alone.

Compound **1,** named ST8161, was prepared according to the following procedure, also drawed in Figure 1. Compound **2** was activated as a chloroformate[18] by treatment with triphosgene, then reacted with Gimatecan[19] to afford carbonate **3**.[20] Removal of Boc protecting group by formic acid gave compound **4** in good yield.[20] (+)-biotin N-succinimidyl ester was prepared according to procedure described earlier.[21] Treatment of **4** with biotin N-succinimidyl ester and TEA in anhydrous DMF afforded compound **1** in 90% yield. Gimatecan was prepared as reported by Dallavalle and co-authors.[22]

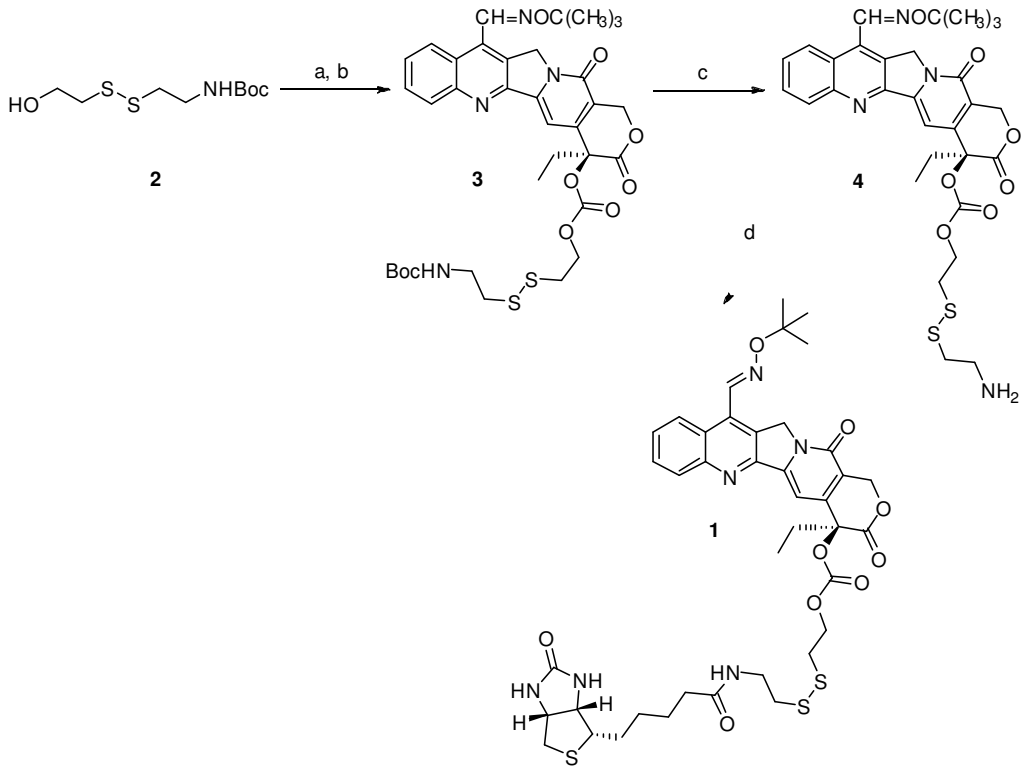

Figure 1. a) triphosgene, Na_2CO_3, diethyl ether/toluene 1:1, 0 °C, 24 h, RT; b) Gimatecan, DIPEA, DMAP, CH_2Cl_2 (dry), 0 °C - RT, 24 h, 50%; c) HCOOH (98%), RT, 1.5 h, 82%; d) biotin-NHS-ester, Et_3N, DMF (dry), 2 h, RT, 90%.

All experimental procedures and complete characterization of compounds are available in the Supporting Information.

To investigate the inhibitory activity of biotinylated Gimatecan ST8161 on tumor cell proliferation, A431 squamous carcinoma cells were exposed to different concentrations of the drug (Fig. 2). The cells in logarithmic phase of growth were seeded in triplicate in 96-well plate at concentration 3000 cells/well in DMEM 10% FBS with Gentamicin. Next day medium was aspirated and the cells were exposed to drug in fresh medium. Cells were incubated for 72 h. Calorimetric assay XTT was applied for analysis of cell proliferation.

Plates were measured at 492nm-690nm with ELISA reader. IC_{50}±SD was defined as the concentration required for 50% cell inhibition as compare to control cells. In two representative experiments IC_{50} value ± SD of ST8161 was 9.25 ± 0.778 nM.

To verify accessibility and binding capacity of biotin in ST8161, competitive ELISA was performed. Multiwell plate (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) was coated on at 4 °C with 100 µl of streptavidin solution at a concentration of 10 mg/mL in 50 mM $NaHCO_3$, pH 9.6. After discarding coating solution, the plate was washed several times with washing buffer (0.05% Tween-20 in PBS) and blocked for one hour at 37 °C with blocking buffer (3% BSA fraction V, 0.05% Tween-20 in PBS). The plate was rinsed and incubated with biotinylated and non biotinylated drugs for one hour at 37 °C. The plate was washed and incubated with biotinylated AP (Sigma). Binding of AP-conjugated biotin was detected by incubation with 1 mg/mL solution of p-nitrophenyl phosphate in substrate buffer (10% diethanolamine buffer, 0.5 mM $MgCl_2$, pH 9.8) for 15 min. The results were expressed as the difference between absorbances at 405 and 620 nm. Development of the plate in presence of non biotinylated Gimatecan and biotinylated ST8161 is shown on Fig. 3. Biotinylated ST8161 efficiently bound streptavidin and blocked development of reaction with biotinylated alkaline phosphatase.

The efficacy of biotinylated toxin ST8161 to inhibit tumor growth *in vivo* in the presence or without AvidinOX was investigated in a mouse tumor xenograft model by using SKOV-3 ovarian carcinoma cell line.

All the procedures adopted for housing and handling animals were in strict compliance with directive 2010/63/UE on the protection of animals used for scientific purposes, made effective in Italy by the Legislative degree 4 March 2014, n. 26, and ARRIVE guidelines.[23] SKOV-3 human ovarian adenocarcinoma cell exponentially growing cells were injected subcutaneously into nude Nu/Nu mice (day zero) ($5x10^6$/mouse in 100 µL) followed by caliper measurements.

AvidinOX was prepared by Areta International in lyophilized form, as previously described (Verdoliva et al, 2010). After reconstitution with sterile water, the protein was 10.0 mg/mL in acetate buffer pH 5.2 with mannitol and NaCl. Small aliquots of AvidinOX solution were stored at -80 °C. For preparation of a single mouse dose of conjugate 10 uL of AvidinOX were mixed with 0.67 µL of 10 mM ST8161 and incubated for 30 min at RT.

On day 18 after tumor cell injection, when tumors were about 100 mm^3, mice were randomized in groups of 8 mice in each and injected with a single intratumor injection of vehicle control (PBS, 10 µL), or ST8161 (6.7 nmol of a 10 mM solution, that is 5.7 µg/mouse or 0.26 mg/kg), or AvidinOX (10 µL of 10 mg/mL solution) or the combination AvidinOX +ST8161 (preincubated for 30 min before the administration). Tumor growth was controlled by a digital Vernier caliper twice a week until sacrifice.

Upon 32 days after the single administration, mice treated with ST8161 showed a significant antitumor activity (TVI=48%, $P<0.05$ vs vehicle treated group), whereas the combination AvOX + ST8161 increased the antitumor effect (TVI= 68%, $P<0.05$ vs vehicle and vs ST8161 alone, Mann-Whitney's test), Fig. 4a. The compounds revealed a good tolerability as shown for body weight (Fig. 4b).

Gimatecan is a synthetic analog of camptothecin, a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I. In our study the ST8161, a biotinylated derivative of Gimatecan, was delivered locally into the site of the human xenograft in murine tumor model in combination with AvidinOX or alone. Our results demonstrated that the best antitumor efficacy was achieved by administration of AvidininOX/biotinylated drug complex. ST8161 bound to four binding sites on AvidinOX

and probably resided inside the tumor for a longer time and was released by degradation of linker. The linker selected for Gimatecan-biotin conjugation was designed to guarantee releasing of cytotoxic drug under acidic (carbonate bond to 20-OH position of CPT) and reducing conditions (disulfide moiety) by GSH, typical of the tumor microenvironment.[24] The half-life of AvidinOX injected into normal tissue and solid tumors was previously shown to be about 14 and 7 days, respectively.[13,25] We assumed that the AvidinOX was continuing anchorage the ST8161 in tumor until the linker between Gimatecan and biotin degraded, thus providing efficient therapy by a single injection. We observed no variation of the weight in ST8161-treated mice as compared to vehicle controls. This observation indicated that general toxicity of ST8161 anchored into the tumor was low, thus making this therapeutic approach very promising for further investigation.

Conflict of interest

Authors are employees of Alfasigma SpA that holds patents on AvidinOX.

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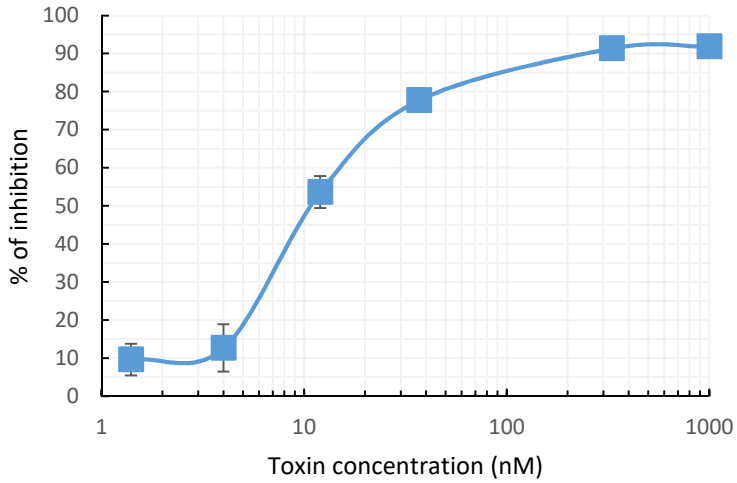

Figure 2. Effect of ST8161 on the growth of A431 cells.

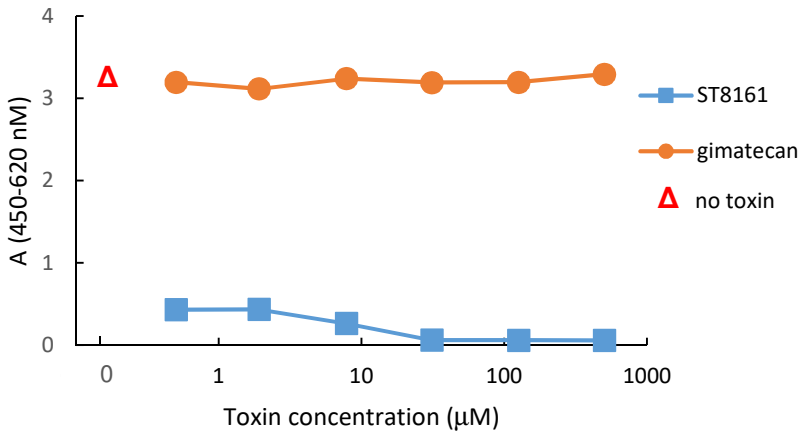

Fig. 3. Competition of biotinylated ST8161 for streptavidin binding. Gimatecan was used as a negative non-biotinylated control.

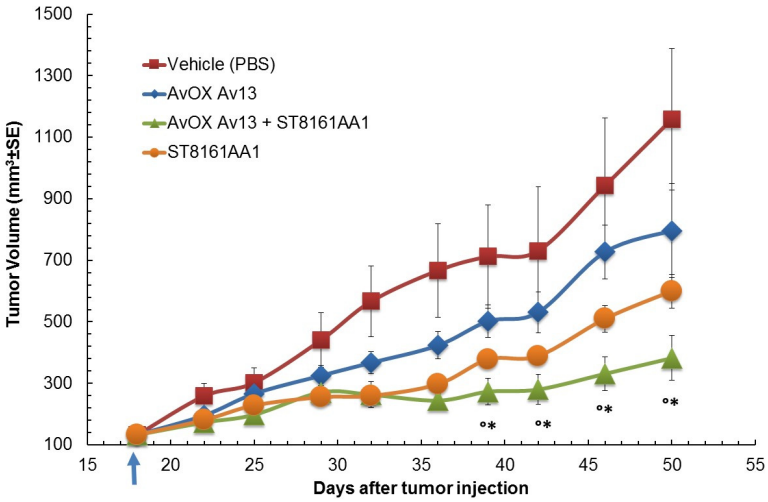

Fig. 4. a) Tumors were allowed to develop in Nu/Nu mice for 18 days after s.c. injection of $5x10^6$ SKOV-3 ovarian tumor cells. Lesion development and response to the biotinylated toxin alone and in combination with AvOX was monitored using a Vernier calyper.

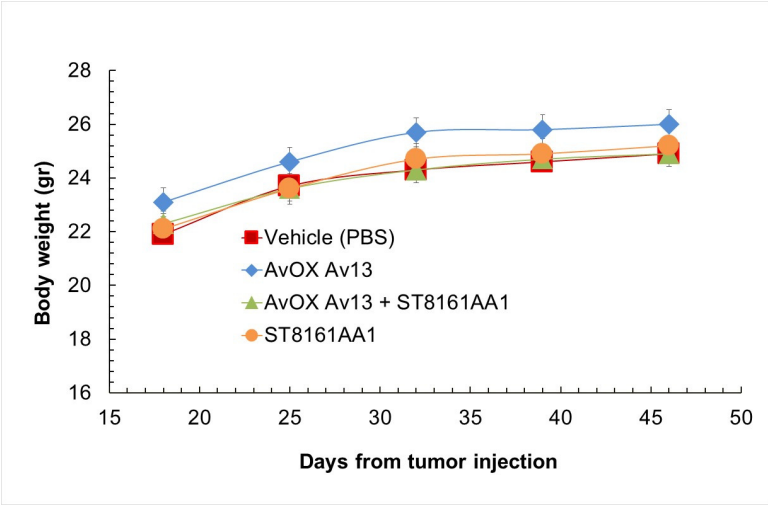

Fig. 4. b) Body weight of SKOV-3 tumor bearing mice, throughout the experiment in a).