Chicken avidin and bacterial streptavidin are widely employed in vitro for their capacity to bind biotin but their pharmacokinetics and immunological properties are not always optimal, thereby limiting their use in medical treatments. Here we investigate the biochemical and biological properties of a new modified avidin, obtained by ligand-assisted sodium periodate oxidation of avidin. This method allows to protect biotin binding sites of avidin from inactivation due to the oxidation step and to delay avidin clearance from injected tissue by generation of aldehyde groups from avidin carbohydrate moieties. Oxidized avidin shows spectroscopic properties similar to that of native avidin indicating that tryptophan residues are spared from oxidation damage. In strict agreement with these results, Circular Dichroism and Isothermal Titration Calorimetry analyses confirm that the ligand-assisted oxidation preserves the avidin protein structure and its biotin binding capacity.

In vitro cell binding and in vivo tissue residence experiments demonstrate that aldehyde groups provide oxidized avidin the property to bind cellular and interstitial protein amino groups through Schiff's base formation, resulting in tissue half life of two weeks, compared to two hours of native avidin. In addition, the efficient uptake of the intravenously injected $^{111}$In-BiotinDOTA (ST2210) in the site previously treated with modified avidin, underlines that tissue bound oxidized avidin retains its biotin binding capacity in vivo.

The results presented here indicate that oxidized avidin could be employed to create a stable artificial receptor in diseased tissues for the targeting of biotinylated therapeutics.

Chicken avidin and bacterial streptavidin are tetrameric proteins containing four identical subunits (homotetramer) each of which can bind to biotin (Vitamin B$_7$, vitamin H) with a high degree of affinity and specificity. The dissociation constant of (strept)avidin-biotin system is measured to be $K_D = 10^{-15}$ M, making it one of the strongest known non-covalent bonds [1]. The high affinity, tetravalency and stability for biotin have made possible to use (strept)avidin-biotin system as probe and affinity tag in a wide variety of applications in the life sciences [2]. These (strept)avidin-biotin techniques are also found to be useful in medical applications in vivo to localize and image cancer cells and to pre-target drugs to tumours [3–6]. In addition, the use of avidin to neutralize the anticoagulant activity of idrabiotaprinux by increasing the plasma clearance of the idrabiotaprinux-avidin complex has been demonstrated [7].

In spite of the fact that avidin and streptavidin are structurally and functionally analogous proteins, they differ in their primary amino acid sequence, pI, glycosylation [8] and immunological reactivity [9]. Avidin contains twice the number of the basic amino acids lysine and arginine compared to streptavidin; therefore the pI of avidin is very high (approx. 10.5), whereas streptavidin has a slightly acidic pI (approx. 6). Each avidin monomer has one N-linked carbohydrate side chain, and approximately 10% of avidin's mass is due to its carbohydrates mannose and N-acetylglucosamine [10,11]. These sugars are not necessary for the stability and the high binding affinity of avidin for biotin [12], but they influence the in vivo avidin biodistribution because of their binding to sugar receptors present throughout the reticuloendothelial system [13-16]. Streptavidin is void of carbohydrates. Owing to these dissimilarities, the plasma half-life and biodistribution of avidin and streptavidin are rather different [17-19]. The plasma half-life of avidin is shorter when compared to that of streptavidin [20,21]. Both the positive net charge and its carbohydrates play a role in avidin’s rapid blood clearance and its accumulation in the liver, spleen and kidneys [22]. Streptavidin, on the other hand, accumulates mainly in the kidney [23]. In addition, avidin and streptavidin, being xenogenic in human, may stimulate, like other xenogenic therapeutic proteins, an immunological response, thus limiting their repeated use [24-26].
To overcome these immunological and pharmacokinetic problems, chemically modified forms of avidin and streptavidin have been developed. Rosebrough and Hartley [19] deglycosylated and chemically lowered the pl of avidin, and attached galactose to streptavidin to modify their pharmacokinetics. Subsequently, Chinol et al. [27] succeeded in reducing the immunological response and increasing the plasma half-life of avidin by its poly(ethylene glycol)ylation and succinylation. Several other recent reports have also described genetically modified or new egg-white avidins, which should offer advantages over native chicken protein [28-34].

In the perspective of improving avidin pharmacokinetic properties for tissue engineering applications, an oxidized avidin has been recently described showing that, once injected in tissues, it might be useful to uptake intravenously injected radioactive biotin [35]. Here we describe in details the procedure to obtain such avidin variant by sodium periodate oxidation of the sugar pyranosydic rings after saturation with the low affinity ligand 4-hydroxyazobenzene-2'-carboxylic acid (HABA) [36]. This reaction produces reactive aldehyde groups (CHO) which are virtually inert at acidic pH, while, when injected in a tissue, due to the physiological pH, react with NH2 groups on tissue proteins, delaying clearance of such modified avidin, named OXavidinHABA. Here it is reported the physico/chemical and biological characterization of oxidized avidins by UV, Size Exclusion Chromatography, Circular Dichroism, Isothermal Titration Calorimetry, Cytofluorimetry and in vivo experiments. Results indicate that the HABA-assisted oxidation substantially preserves the structure and the biotin binding property of native avidin which acquires the capacity to reside within injected tissues for weeks as consequence of the chemical reaction of aldehyde groups with tissue protein amino groups by Schiff’s base formation. The use of native avidin, injected during surgery in the tissue surrounding an excised breast tumor, followed by intravenous radioactive biotin within the next 24 hours, has been recently applied in the clinic to prevent local tumor recurrences. This method, named Intraoperative Avidination for Radionuclide Treatment (IART®) [37-39], is an innovative way to perform pre-targeted tissue radiotherapy but it is conditioned by the short tissue half-life of native avidin that, diffusing from the injected breast into liver and kidney, requires the blocking of the radioactive biotin uptake in these organs that is performed by injecting human biotinylated albumin (HSAbiot), 10 minutes before radioactive biotin. Present data confirm that OXavidinHABA, being stable in injected tissues, could be a valid alternative to native avidin in IART®. Moreover, it could be employed for other medical applications where a stable artificial receptor in diseased tissues could be envisaged to direct biotinylated therapeutics.

**EXPERIMENTAL PROCEDURES**

_Avidin oxidation_ - Native chicken avidin (BioSPA) was incubated, at the final concentration of 3 mg/mL, with sodium periodate, varying from 0 to 40 mM, to oxidize sugars as previously described [40] in 100 mM acetate buffer at pH 5.5 for 1 hour at room temperature in the presence or absence of 4'-hydroxyazobenzene-2-carboxylic acid (HABA) (Sigma). At the end of incubation oxidized avidin was loaded on a Sephadex G-25 Fine Desalting column (GE Healthcare), and formulated in 100 mM acetate buffer, pH 5.5. To provide complete removal of sodium periodate and HABA, a maximum sample volume of 5% of the total column volume is recommended.

The amount of aldehyde groups generated by oxidation of the sugars was evaluated using the reaction of aldehydes with Purpald® (4-amino-3-hydrazino-5-mercapro-1,2,4-triazole,4-amino-5-hydrazino-4H-1,2,4-triazole-3-thiol), described by Quesemberry and Lee [41]. Blocking of CHO groups was performed by reacting avidin with 50-200 mM semicarbazide (Sigma). The biotin binding activity of avidin was measured according to the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) method [36].

**Isothermal Titration Calorimetry (ITC)** - All avidins were extensively dialyzed against 100 mM acetate buffer, pH 5.5, the identical buffer in which ST2210 (biotinDOTA) [42] was solubilized, and all solutions were degassed prior to use. Titrations were carried out using 10 µL injections given every five min. The concentration of ST2210 in the syringe was 18-25 times the concentration of the avidin in the cell: 2.4-2.8 mM ST2210 was titrated into 0.11-0.13 mM avidin. All titration data were collected at 25 °C and replicated to determine the experimental standard deviation for each parameter. Binding isotherms were fitted by nonlinear regression using the single-site model provided by Origin software (MicroCal, Inc.). The stoichiometry of the interaction (N), equilibrium association constant (K_a), and change in enthalpy (ΔH) were floated...
Spectroscopic analyses - Absorbance spectra, in the UV range 240-300 nm, were recorded for native and oxidized avidins on a Beckman DU 640 spectrophotometer. Samples were analyzed in 100 mM sodium acetate buffer, pH 5.5, at a final concentration of 0.4-0.5 mg/mL. Far-UV CD spectra of native and oxidized avidins were recorded in 100 mM sodium acetate buffer, pH 5.5, with or without 4 equivalents of biotin, at 25°C and at a 4.5 µM concentration using a 1 mm path length quartz cell. Spectra, from 190 to 260 nm, were acquired by Jasco J-715 spectropolarimeter equipped with a thermostatic water bath. Each spectrum was obtained averaging three scans and subtracting the contribution from the buffer solution. Other experimental settings were: 20 nm×min⁻¹ scan speed, 0.2 nm band width, 0.2 nm resolution, 50 mdeg sensitivity, and 4 sec response. Melting curves were recorded by following the decrease of dichroic signal at 225 nm meanwhile increasing the temperature, in the range 25-95°C, with the following instrument settings: band width 1 nm, response 0.5 sec, data pitch 1°C, temperature slope 10°C/min. Moreover, at every temperature increment of 5 Celsius degrees a far-UV CD spectrum was collected in the experimental conditions reported above. The point of inflection and slope (p) of sigmoidal curves were calculated by means of Boltzman fitting model of Origin® 7.0 software.

Cell lines and FACS analysis - Avidin and streptavidin binding to cells was analyzed by flow cytometry on human tumor cell lines U118MG (glioma) and PC3 (prostate) or murine fibroblasts (3T3), mouse spleen and red blood cells. Cells were incubated at 37°C for 2 hours in the presence of avidin or streptavidin (5µg/5x10⁵ cells in 100 µL of PBS) and after washings, the binding was detected by biotin-B-PE (Fluka) 2µg in 100 µL of PBS, 1 hour at 4°C. Background was obtained by incubation of cells with biotin-B-PE only. Analysis was performed on a FACScalibur instrument and data elaborated by CellQuest software (Becton Dickinson).

Animals - The care and husbandry of mice were in accordance with the European Directive 86/609 and Italian legislation.

Residency of (strept)avidins in tissue and uptake of ¹²⁵I-ST2210 - Female Balb/c nude mice (Charles River) were used. The animals were divided in groups of 5 mice each. ¹²⁵I radiolabeling of avidin and streptavidin (both from Biospa) was performed with ¹²⁵Iodine by IODO-GEN™ tubes (Pierce) before periodate oxidation since iodination reaction occurs at pH 7.5 at which intermolecular formation of Schiff’s bases of oxidized glycoproteins would occur. ¹²⁵I-labeled avidin, streptavidin or oxidized avidin were injected in one hind limb (tissue avidination) at the dose of 50 µg in approximately 15 µL of 100 mM sodium acetate pH 5.5, corresponding to 6 x 10⁵- 2 x10⁶ cpm. Groups of mice were sacrificed at the indicated time points. The injected limb and blood, liver, kidney and contralateral limb were collected, weighted and radioactivity quantified by gamma counter (Canberra Packard). Since the weight of the treated tissues was less than 1 g, data were expressed as the % of injected dose/100 mg (% ID/100mg) of tissue and are the average of 5 animals +/- standard deviation (+/- SD). For ¹¹¹In-biotin uptake, mice were avidinated as described above and injected intravenously with 16 µg of ¹¹¹In-ST2210 at the indicated time points. For reducing background, some groups of mice received an intravenous bolus (80 µg) of biotinylated human serum albumin (HSAbiot) to block radioactivity uptake in non target tissues. This chase step was performed 10 minutes before ¹¹¹In-ST2210 intravenous administration. All mice were sacrificed 1 hour after ¹¹¹In-ST2210 injection and the avidinated limb, blood, liver, kidney and contralateral limb were collected, weighted and radioactivity quantified by gamma counter (Canberra Packard). Data were expressed as the % of injected dose/g (% ID/g) of tissue and are the average of 5 mice +/- standard deviation (+/- SD).

RESULTS

Cell binding and tissue kinetics of avidin and streptavidin. Looking for a stable receptor for radiolabeled biotin, in tissues to be exposed to radiotherapy, preliminary experiments were performed to investigate the in vitro cell binding and in vivo tissue residence and efficiency in capturing the ST2210 radioactive biotin of both reference molecules avidin and streptavidin. As shown in Fig. 1a, and as expected from bibliographic data [43], binding of avidin but not streptavidin to a panel of tumor as well as normal cells was confirmed by FACS analysis. Therefore, to evaluate their diffusion kinetics from a treated tissue, either ¹²⁵I-radiolabelled avidin and streptavidin were injected in the muscle of one hind limb of mice simulating the recently described approach of cancer pre-targeted radiotherapy named IART® [37-39], and at different time points mice were sacrificed and the...
treated and controlateral limb (Muscle), blood, liver and kidney were collected and radioactivity was measured by gamma counter. Previously published data from our group, showed that the residence time of $^{125}$I-labelled avidin and streptavidin in the treated tissue is very short, with approximately 8% of the injected dose/100 mg observed after 1 hour, and less than 1 % ID/100 mg after 24 hours. In agreement to previous pharmacokinetics data on avidin and streptavidin intravenously injected, the avidin diffused from the injected tissue, exhibited a faster clearance from the blood than streptavidin and it accumulated in the liver and kidney, while streptavidin preferentially accumulated in the kidney [35].

Taking into account such diffusion kinetic and biodistribution data the uptake of radioactive biotin was evaluated in the same mice pre-injected with avidin or streptavidin. $^{111}$In-ST2210 was intravenously administered 1 or 24 hours after tissue avidination, with or without a chasing step with HSAbiot, performed to reduce radioactivity localization in non avidinated tissues. The Fig. 1b (top panel) shows that, after 1 hour from avidin or streptavidin intramuscular injection, the highest $^{111}$In-ST2210 localization is in the avidin or streptavidin pre-injected limb and kidney. Blood and controlateral limb (Muscle) contain less than 0.2% ID/g of tissue, except for the blood of animals treated with streptavidin that contains about 1.5% ID/g of $^{111}$In-ST2210 as a consequence of the slower clearance of streptavidin compared to avidin. A clear effect of the HSAbiot chase can be observed in non target organs where the non specific uptake of radioactive $^{111}$In-ST2210, due to the presence of either streptavidin or avidin diffused from the treated limb, is quenched. It is to point out that the selected chasing condition does not affect the specific radioactivity localization in the treated limb as also shown in the IART® clinical study [38].

In agreement with data of tissue residence, 24 hours after tissue avidination the uptake of $^{111}$In-ST2210 was very low. In the avidinated limb, as well as in blood, liver and controlateral limb the localization of $^{111}$In-ST2210 was below 0.5 % ID/g of tissue (Fig. 1b bottom panel). A higher radioactivity signal was observed in the kidney of the mice treated with streptavidin compared to avidin as a consequence of streptavidin slower elimination from the circulation.

Oxidized avidins production and characterization. In an attempt to improve the tissue half-life of avidin while reducing its localization in non target organs, and taking into account that avidin oligosaccharides are not essential for biotin binding, native avidin was chemically modified in the sugar moieties by oxidation with sodium periodate according to the scheme in Fig. 2a. This reaction has been widely used from the early 70’ to conjugate in vitro glycoproteins to functional protein moieties [40,44]. At acidic pH, the aldehyde groups (CHO) are substantially inert because protein amino groups (pKa = 8-9) are in the protonated NH$_3^+$ status but at neutral pH and higher, aldehyde groups react with protein amino groups to form Schiff’s bases.

In a preliminary study performed in a mouse model simulating pre-targeted radiotherapy in the muscle, avidin was oxidized with sodium periodate and formulated at pH 5.5 to obtain oxidized avidin. Equal amount of $^{125}$I-labeled oxidized avidin or avidin were then intramuscularly injected in one hind limb of mice and their tissue permanence evaluated at different time points. One hour after injection, the amount of oxidized avidin in the treated limb was twice that of avidin, and the difference increased with time: avidin was almost undetectable after 24 or 48 hours while oxidized avidin was always higher than 10% ID/100 mg of tissue [35]. These findings suggested that oxidation allows the avidin to react with tissue proteins in vivo by the formation of Schiff’s bases.

To investigate the effect of CHO groups on tissue binding and to optimize the oxidation reaction in terms of oxidized avidin (OxidAV) stability and aldehyde groups production, experiments were performed by varying the periodate concentration from 1 to 40 mM allowing it to react for 1 hour at room temperature. Data, collected in Table 1, panel a, indicate that the amount of CHO groups generated by oxidation, and the % ID/100 mg of tissue of OxidAV, after 24 hours from injection, increased with the periodate concentration, thus suggesting that the tissue residence of avidin is strictly related to the number of CHO produced. To prove this relationship, $^{125}$I-labeled OxidAV (20 mM periodate concentration) was reacted with semicarbazide to block the aldehyde groups, according to the following reaction:

$\text{avidin-CHO + NH}_2\text{NHCONH}_2 -> \text{avidin-CH=NNHCONH}_2$

Tissue permanence of OxidAV and 50 or 200 mM semicarbazide derivatives, 24 hours after intramuscular injection in limb as previously described, was about 16, 6 and 2 % ID/100 mg, respectively, indicating a correlation of tissue...
residence with the number of CHO that were 13, 5 and 2 per molecule, respectively (Fig. 2b). Nevertheless, gel filtration analyses of oxidized avidins (not shown) indicated that for periodate concentration over 20 mM protein degradation occurred with significant reduction of its biotin binding capacity, as also determined by HABA test performed with ST2210.

Considering that the tissue permanence of avidin oxidized with 10 and 20 mM NaIO₄ at 24 h was about 8-10 times that of avidin, lower or higher periodate concentrations were also tested but discarded for further studies for being either not adequate to generate sufficient CHO groups or too severe to maintain acceptable level of avidin biological activity.

As previously reported [45], the oxidation with sodium periodate of the sugar pyranosidic rings of avidin is associated to some oxidation of tryptophan aminoacidic residues which are known to be important for theavidin binding, thus explaining the reduced ST2210 binding properties of such oxidized avidin. In order to prevent the damage of the biotin binding site, the avidin oxidation was performed in the presence of saturating amount of the low affinity ligand 4-hydroxyazobenzene-2’-carboxylic acid (HABA). As shown in Table 1, panel b, the protective effect of HABA in avidin oxidation is confirmed by data of ST2210 binding that show for avidin oxidized with 10 or 20 mM sodium periodate, in the absence (OxidAV) or in the presence of HABA (OXavidinHABA), values of about 50 and 80%, respectively compared to about 100% binding capacity.

Data also indicate that the number of CHO groups generated by oxidation and the avidin tissue permanence after 24 hours, increased with the periodate concentration, thus confirming that the tissue permanence of oxidized avidins is strictly related to the number of CHO groups produced.

The oxidized avidins were further characterized for their biochemical and biophysical features.

As reported by Green [45], the extent of tryptophan oxidation is strictly related to the lowering of the characteristic inflexions at 282 and 291 nm, while the absorbance increases in the 250-260 nm region, due to the formation of a substituted oxindole. Analysis of the absorption spectra of OxidAV and OXavidinHABA, compared to that of the unmodified avidin (Fig. 3a), indicated that combination with HABA greatly diminished the rate of tryptophan oxidation. The size exclusion chromatography analysis of avidins confirmed these results. Concentrations being equal, OxidAV, due to more extensive protein degradation, showed the shortest peak, with an absorbance 280/260 ratio of about 1.0, compared to 1.5 of OXavidinHABA and 1.7 of avidin. In addition, the elution time of oxidized avidins appeared slightly delayed compared to that of avidin, most likely due to modification of hydrodynamic behaviour consequent to oligosaccharides oxidation (Fig. 3b).

In strict agreement with these findings, OXavidinHABA showed structural and thermodynamic properties very similar to avidin. Thermal stability and conformational changes were determined by circular dichroism spectroscopy before and after heating (Fig. 4, top and bottom panel, respectively), without and with 4 equivalents of biotin (Fig. 4, panel a and b, respectively). Data, collected in Table 2, indicated that oxidation decreased thermal stability, as determined by the lowering of the melting temperature (Tₘ) and of the slope (p) of the sigmoidal curve of OxidAV compared to that of native avidin (74.3 versus 79.0 and 8.9 versus 14.7 °C, respectively). Nevertheless, the destabilization effect of oxidation was almost totally inhibited by incubation of avidin with HABA, as confirmed by the Tₘ and p values of OXavidinHABA and avidin, corresponding to 78.1 versus 79.0 and 11.2 versus 14.7 °C, respectively. While thermal denaturation was irreversible for both OxidAV and OXavidinHABA when heated without biotin only OXavidinHABA retrieved its secondary structure, similarly to avidin, when heated/cooled in the presence of biotin.

These findings confirm that the occupancy of biotin binding sites by HABA preserved the conformation of avidin subjected to chemical oxidation and explain the consequently retained biotin binding capacity.

Evaluation of thermodynamic parameters determined by titration of avidins with ST2210, by means of Isothermal Titration Calorimetry (ITC) (Fig. 5), underlines that ST2210 was able to bind to OXavidinHABA and avidin (left and right panels, respectively) in a comparable manner, as the association constants (Kₐ) and enthalpy change (ΔH) were of the same magnitude while the ST2210/OxidAV interaction showed, as expected, lower Kₐ and higher ΔH (Table 3). According to data of ST2210 binding to oxidized avidins, within experimental error, the determined stoichiometry of interaction is 3.0, 1.2 and 2.5 molecules of ST2210 per molecule of avidin, OxidAV and OXavidinHABA, respectively.

Residency of oxidized avidin in tissue and uptake of 111In-ST2210. In order to evaluate the
potential of oxidized avidin for therapeutic applications, the chemical hypothesis that this modified avidin would interact with tissue proteins and remain longer than native avidin in a treated tissue was further investigated by injecting $^{125}$I-labeled avidin or OXavidin$_{HABA}$ in the muscle of one hind limb of mice, as previously described. At the indicated time points, mice were sacrificed to count radioactivity in the treated limb as well as in other non target organs.

Results in Fig. 6 indicate that the half-life of OXavidin$_{HABA}$ in the treated limb is about 2 weeks as opposed to about 2 hours previously reported for avidin [35]. Both avidins were almost undetectable in blood, liver and kidney after two days from the intramuscular injection (data not shown).

The significantly higher tissue residence of OXavidin$_{HABA}$ compared to avidin in the injected limb is paralleled by a higher uptake of $^{111}$In-ST2210 injected intravenously 16 or 48 hours after the treatment with avidin and OXavidin$_{HABA}$. Groups of mice were sacrificed 2, 24 and 72 hours after the injection of $^{111}$In-ST2210 and the radioactivity in the treated limb measured by gamma counter. Data in Figure 7 show that OXavidin$_{HABA}$-treated limbs exhibited a highly efficient uptake of radiolabeled biotin, both when the radiolabeled biotin was injected 16 (upper panel) or 48 (lower panel) hours after tissue avidination, and that the binding was stable. In the same conditions the uptake of $^{111}$In-ST2210 by native avidin-treated limbs was almost negligible. The Area Under the Curve (AUC) of this study, calculated taking into account the loss of energy due to the spontaneous radioactive decay of $^{111}$In, after 16 and 48 hours were 19.2 versus 529.8 and 11.8 versus 498.6 for avidin and OXavidin$_{HABA}$, respectively.

Considering that polyethylene glycol (PEG) conjugation of avidin was previously attempted to improve its pharmacokinetic and biodistribution properties [27,46], and to investigate the extraordinary tissue binding properties of oxidized avidin, $^{125}$I-PEG-avidin (hydrazone) was previously produced by reacting CHO groups of $^{125}$I-oxidized avidin with a molar excess of 10 kDa PEG-hydrazide. After purification by size exclusion chromatography, $^{125}$I-PEG-avidin, was injected in the muscle of mice as previously described and its tissue residence was compared to the one of radiolabeled avidin and oxidized avidin. Results indicated that, 24 hours after injection, less than 2% ID/100 mg of PEG-avidin and avidin was present in the treated limb versus about 16 % of oxidized avidin [35]. These findings, besides ruling out the possibility to improve the tissue permanence of avidin simply by increasing its size as for intravenously injected PEG-avidin, provide further evidence of the relationship between oxidized avidin tissue permanence and aldehyde groups.

**DISCUSSION**

With the aim of promoting a stable binding of avidin to target tissues, we have previously investigated the feasibility to exploit the sugar pyranosidic rings of avidin to generate reactive groups [35]. Therefore, according to the well known and specific method which allows the generation of reactive aldehydes on the carbohydrate moieties of proteins by oxidation with sodium periodate [40,44], native avidin was chemically modified by means of HABA-assisted oxidative cleavage of oligosaccharides. The interaction of avidin with the benzoate ring of HABA is relatively weak ($K_d = 5.8 \times 10^{-6}$ M) but, since it emulates the high-affinity interaction of the protein with the ureido moiety of D-biotin ($K_d = 1 \times 10^{-15}$ M), the avidin/HABA complex formation preserves, from the oxidation damage, the protein structure and its biotin binding capacity. This oxidized avidin, named OXavidin$_{HABA}$, exhibits CHO groups substantially inert when the product is formulated at acidic pH, but which react with NH$_2$ groups at neutral pH to form bases of Schiff. Interestingly, the formation of Schiff’s bases involving oxidized avidin occurs with living cells and tissue proteins, and this interaction results in a tissue half life of two weeks, compared to two hours of native avidin. The overall biochemical profile of OXavidin$_{HABA}$ describes a product, free or complexed to biotinylated therapeutics [35] exhibiting, by Purpald’s method, 8-15 active CHO groups/molecule, generated by oxidation with sodium periodate.

Regarding the biochemical characterization, OXavidin$_{HABA}$ shows spectroscopic properties similar to that of native avidin, indicating that tryptophan residues are spared from oxidation damage. In strict agreement with these results, Circular Dichroism and Isothermal Titration Calorimetry analyses confirm that HABA-assisted oxidation prevents the destabilization effect due to the periodate oxidation, as demonstrated by the highly efficient binding of biotin.

A lot of effort has been recently devoted to the development of targeted delivery systems for brachytherapy (brachios, in Greek meaning short, refers to radiotherapy of tumors by contact),
including biodegradable dextran or chitosan hydrogels [47,48]. The avidin property of spontaneous association to boric acid gel suspension has been exploited to promote binding of avidin to tumor cells in vivo [49]. A fusion protein named Scavidin consisting of the macrophage scavenger receptor class A and avidin, that was shown to be binding and being internalized by tumor cells [50], an avidin/biotin liposome system for peritoneal delivery [51] and avidin bioconjugate with thermoresponsive polymer [52] were also previously described.

The use of oxidized avidin may offer a number of potential advantages also compared to current brachytherapy devices like permanent radioactive seeds [53,54], catheters [55,56], balloons [57, 58] or other modified avidins. For instance, the perfusion of a target tissue with OXavidinHABA could allow to delay of several days the administration of biotinylated therapeutics whenever the patient conditions or the logistics might recommend it, or to fractionate the therapeutic dose with no need to immobilize the treated tissue, as for example, in current seeds brachytherapy to prevent migration of the seeds. Moreover, as most of the injected OXavidinHABA binds and resides in the treated tissue, it will be not necessary to perform a chasing step with biotinylated albumin to block biotin uptake in non target organs.

It should also be taken into consideration that the formation of Schiff’s bases between oxidized sugars and protein NH2 groups is a common event in vivo, leading to protein glycation [59]. Moreover, hundreds of oxidized proteins have been recently characterized in plasma of animals as the common product of oxidative stress [60,61]. In addition, it is noteworthy that the use of HABA for the preparation of OXavidinHABA for human use is convenient because such low affinity, non-toxic ligand can be easily removed by simple dialysis or chromatography. Pre-clinical safety and immunogenicity studies with OXavidinHABA are ongoing in both rodents and non human primates showing good local and systemic tolerability (manuscript in preparation).

On the overall, we believe that OXavidinHABA is a promising device that, for its peculiar features, exhibits an extraordinary potential for a variety of therapeutic applications if thought in combination with different biotinylated agents like radioisotopes, chemotherapeutics, growth factors, plasmids, viral vectors, effector or stem cells. Therefore, besides cancer, OXavidinHABA might be employed for directing biotinylated therapeutics to different tissues for the cure of inflammatory, autoimmune, degenerative or genetic diseases.

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**FOOTNOTES**

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The abbreviations used are: IART®, Intraoperative Avidination for Radionuclide Treatement; HSAbiot, biotinylated human serum albumin; HABA, 4'-hydroxyazobenzene-2-carboxylic acid; OxidAV, oxidized avidin; OXavidinHABA, HABA-protected oxidized avidin.

**FIGURE LEGENDS**

Fig. 1. *In vitro* cell binding and *in vivo* uptake of $^{111}$In-ST2210 by avidin and streptavidin. Panel a: Flow cytometry of human prostate cancer PC3, mouse fibroblast NIH3T3, human glioma U118MG, mouse spleen and red blood cells. Cells were incubated at 37°C for 2 hours in the presence of avidin (unbroken line) or streptavidin (dotted line) and the binding detected by biotin-B-PE. Grey peaks represent the background. Panel b: uptake of intravenously administered $^{111}$In-ST2210 by avidin or streptavidin 1 (top) or 24 (bottom) hours after their intramuscular injection in a limb with or without HSAbiot chase. Mice were sacrificed 1 hour after $^{111}$In-ST2210 injection. The highest uptake of radiolabeled biotin is evident in the treated limb at 1 hour but not at 24 hours. HSAbiot chase reduces background in non target organs. Uptake in controlateral untreated limbs is also indicated (Muscle). Data are the average of 5 mice and bars represent standard deviation.

Fig. 2. Panel a: chemical strategy for oligosaccharide oxidation by sodium periodate and Schiff’s base formation. Panel b: dependence of OxidAV tissue residence on reactive aldehyde groups generated by oxidation with sodium periodate. Tissue residence of oxidized avidin (white) is reduced by reduction of its number of CHO groups (gray) with 50 or 200 mM semicarbazide. Evaluation was performed 24 hours after injection.

Fig. 3. Panel a: absorption spectra of OxidAV and OXavidinHABA, compared to that of the unmodified avidin, in the UV range 240-300 nm. UV spectrum of OXavidinHABA is similar to that of avidin while that of OxidAV exhibits increased absorbance in the 250-260 nm region, due to the formation on tryptophan of a substituted oxindole. Panel b: size exclusion chromatography analysis of avidin, OxidAV and OXavidinHABA on a BiosepSEC S3000 column. Peaks of oxidized avidins appear slightly delayed compared to the one of avidin, most likely due to modification of hydrodynamic behaviour consequent to oligosaccharides oxidation.

Fig. 4. Circular Dichroism spectra of native and oxidized avidins. Panel a: CD spectra, recorded at 25°C (top), show that OxidAV, and OXavidinHABA lack their secondary structure when heated from 25 to 95°C.
(bottom), if compared to avidin. On the contrary, addition of D-biotin (panels b) stabilizes OXavidin$_{HABA}$ and avidin upon thermal denaturation (bottom) unlike OxidAV.

**Fig. 5.** Calorimetric titration of OXavidin$_{HABA}$ (top panel a) and avidin (top panel b) with ST2210 in sodium acetate 100 mM, pH 5.5. The heat of dilution of ST2210 into buffer was subtracted. Bottom panels a and b: enthalpy per mole of ST2210 injected versus ST2210/OXavidin$_{HABA}$ and avidin molar ratio, respectively.

**Fig. 6.** Long term kinetic of $^{125}$I-labeled avidin (○) and OXavidin$_{HABA}$ (■) after intramuscular injection in the limb of Balb/c mice. At indicated time points mice were sacrificed and samples of treated limb were weighted and counted in a gamma counter. Data are expressed as the % of injected dose/100 mg (% ID/100mg) of tissue. Each point corresponds to the average of 5 mice. Bars represent standard deviation.

**Fig. 7.** Uptake of $^{111}$In-ST2210 in the muscular limb pre-treated 16 (panel a) or 48 (panel b) hours earlier with avidin (○) or OXavidin$_{HABA}$ (▲). Mice were sacrificed 2, 24 or 72 hours after intravenous injection of $^{111}$In-ST2210 and samples of treated limb weighted and counted in a gamma counter. Data are expressed as the % of injected dose/g (% ID/g) of tissue. Each point is the average of 5 mice. Bars represent standard deviation. This result confirms OXavidin$_{HABA}$ tissue stability and shows its capacity to uptake and keep bound the radioactive biotin.
|                | NaIO$_4$ mM | CHO/avidin molar ratio ± SD | ST2210 binding % ± SD | Tissue residence 24h % ID ± SD |
|----------------|-------------|-----------------------------|------------------------|-------------------------------|
| Avidin         | -           | <LoQ                        | 100*                   | 2.1 ± 0.4                     |
| (a)            |             |                             |                        |                               |
| 1              | 5.3 ± 0.6   | 75.0 ± 0.7                  | 3.1 ± 0.7              |                               |
| 5              | 7.7 ± 0.7   | 54.2 ± 2.3                  | 6.3 ± 0.3              |                               |
| OxidAV         | 10          | 8.6 ± 1.3                   | 49.7 ± 2.2             | 16.0 ± 0.2                    |
|                | 20          | 12.1 ± 1.3                  | 47.7 ± 2.2             | 19.2 ± 1.7                    |
|                | 40          | 9.8 ± 1.8                   | 43.0 ± 1.0             | NT                            |
| (b) OXavidinHABA | 10        | 9.3 ± 1.0                   | 84.0 ± 1.4             | 16.2 ± 2.3                    |
|                | 20          | 14.2 ± 2.2                  | 79.3 ± 1.1             | 19.0 ± 0.2                    |

SD = Standard Deviation  
NT = Not Tested  
* The experimental value of 97.4 ± 0.5% obtained with ST2210 compared to free biotin by HABA assay is assumed as 100% reference value for modified avidins.  
Data are representative of at least three independent determinations.
Table 2. Thermal stability comparison of avidin, OxidAV and OXavidinHABA determined by circular dichroism spectroscopy before and after oxidation, with and without 4 equivalents of biotin.

|               | Tm °C | p*     |
|---------------|-------|--------|
| Avidin        | 79.0 ± 0.1 | 14.7 ± 0.3 |
| Avidin + 4eq biotin | > 95 | n.a.    |
| OxidAV        | 74.3 ± 0.1 | 8.9 ± 0.1  |
| OxidAV + 4eq biotin | 86.3 ± 0.2 | 20.7 ± 1.1 |
| OXavidinHABA  | 78.1 ± 0.3 | 11.2 ± 0.4 |
| OXavidinHABA + 4eq biotin | > 95 | n.a.    |

*Slope of sigmoidal curve; n.a. = not applicable

Table 3. Thermodynamic parameters comparison of avidin, OxidAV and OXavidinHABA interaction with ST2210, determined by Isothermal Titration Calorimetry (ITC).

|               | N      | K M⁻¹ | ΔH kcal mol⁻¹ | ΔS cal mol⁻¹ K⁻¹ |
|---------------|--------|-------|----------------|------------------|
| Avidin        | 3.0 ± 0.016 | 3.45E6 ± 3.07E5 | -1.48E4 ± 114.0 | -19.6            |
| OxidAV        | 1.2 ± 0.012  | 6.45E5 ± 5.69E4   | -0.79E4 ± 117.8  | 0.16             |
| OXavidinHABA  | 2.5 ± 0.015  | 2.69E6 ± 2.74E5   | -1.163E4 ± 98.4  | -9.60            |
Fig. 1

(a) Flow cytometry histograms showing cell surface expression of the antigen in different cell lines: PC3, 3T3, U118MG, and Spleen Cells. The graphs display the fluorescence intensity (FL2H) against the cell number.

(b) Bar graphs illustrating the percentage of antibody binding (antibody-streptavidin conjugate) in different tissues: Limb, Blood, Kidney, Liver, and Muscle. The graphs show the percentage of bound antibody under 'No Chase' and 'H3HbCH' conditions.
Fig. 2

(a) Molecular structures:

\[ \text{CH}_2\text{OH} \quad \text{NaIO}_4 \quad \text{CH}_2\text{OH} \quad \text{R}-\text{NH}_2 \]

\[ \text{O} - \text{C} - \text{O} \quad \text{O} - \text{C} - \text{N} - \text{R} \quad \text{O} - \text{C} - \text{N} - \text{R} \]

\( \alpha \)-D-Mannose

Schiff's bases

(b) Bar chart:

- % ID/100 mg
- CHO/Oxidin molar ratio

OxidAV
OxidAV/Semicarbazide
50 mM
OxidAV/Semicarbazide
200 mM

Legend:
- OxidAV
- OxidAV/Semicarbazide
- 50 mM
- 200 mM
Fig. 4
Fig. 6
Fig. 7

(a) Graph showing the percentage ID/g over time after ST2210.

(b) Graph showing another set of data points over time after ST2210.

Time after ST2210:

+2h | +24h | +72h
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