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*Research Article*

# OXavidin for Tissue Targeting Biotinylated Therapeutics

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Avidin is a glycoprotein from hen egg white that binds biotin with very high affinity. Here we describe OXavidin, a product containing aldehyde groups, obtained by ligand-assisted sugar oxidation of avidin by sodium periodate. OXavidin chemically reacts with cellular and tissue proteins through Schiff's base formation thus residing in tissues for weeks while preserving the biotin binding capacity. The long tissue residence of OXavidin as well as that of OXavidin/biotinylated agent complex occurs in normal and neoplastic tissues and immunohistochemistry shows a strong and homogenous stromal localization. Once localized in tissue/tumor, OXavidin becomes an "artificial receptor" for intravenous injected biotin allowing tumor targeting with biotinylated therapeutics like radioisotopes or toxins. Moreover, present data also suggest that OXavidin might be useful for the homing of biotinylated cells. Overall, OXavidin exhibits a remarkable potential for many different therapeutic applications.



## 1. Introduction

Selective targeting of diseased tissues has been a challenging goal for decades. However, especially for cancer, no simple solution has been found that might constitute a valid alternative to surgery. Brachytherapy is currently being recognized as an optimal way for delivering conformal radiotherapy to inoperable tumors [1] or oligometastases [2]. Nevertheless, current brachytherapy must rely on a wide armamentarium of devices including balloons [3], catheters [4], or permanent seeds [5] whose placement and accurate dosimetry imply sophisticated and time consuming processes. Recently, a novel pretargeted brachytherapy method, named Intraoperative Avidination for Radionuclide Treatment-IART®, has been described in patients subjected to surgery for early breast cancer to prevent local recurrences [6]. This approach is based on the intraoperative injection of avidin in the surgical margins surrounding the excised tumor followed by the intravenous injection of radioactive biotin within the subsequent 24 hours. Avidin is a basic glycoprotein of about 66 kDa extracted from hen egg white known for its high affinity for biotin [7, 8]. It is composed of four subunits of identical amino acid sequence, each of which can bind one molecule of biotin. Glycosylation accounts for about 10% of its molecular weight with an average of four to five mannose and three N-acetylglucosamine residues per subunit [9]. The short blood and tissue half life of avidin was previously reported [10, 11]. In the present study we chemically oxidized the avidin sugars with sodium periodate generating aldehyde groups which proved able to react with tissue protein amino groups in vivo. To prevent damage to the biotin binding sites, avidin oxidation was performed after saturation with the low affinity ligand 4-hydroxyazobenzene-2′-carboxylic acid (HABA) [12] which is easily removed at the end of oxidation. Such product named OXavidin exhibits the property to stably bind normal and neoplastic tissues through Schiff's base formation. A proof of principle of its use in highly efficient brachytherapy and preliminary data showing its capacity to bind biotinylated cells are here reported.

## 2. Materials and Methods

*2.1. Periodate Oxidation of Avidin.* Avidin (Tecnogen SpA, Caserta, Italy) was incubated, at the final concentration of 3-4 mg/mL, with different concentrations of sodium periodate ($NaIO_4$) (Sigma-Aldrich, St Louis, USA), in 100 mM acetate buffer at pH 5.5 for 1 hour at room temperature, with or without a molar excess of 4-hydroxyazobenzene-2′-carboxylic acid (HABA) (Sigma-Aldrich) obtaining OXavidin ($OXavidin_{HABA}$) or oxidized avidin, respectively. At the end of incubation OXavidin was freed from HABA and formulated, like avidin and oxidized avidin, in 100 mM acetate buffer, 150 mM NaCl, pH 5.5, by diafiltration. The number of aldehyde groups (CHO), generated on the carbohydrate moieties by oxidation, was evaluated using Purpald reagent (Sigma-Aldrich) and propionaldehyde as standard. The biotin-DOTA (ST2210) [13] binding capacity of OXavidin, oxidized avidin, and PEGylated avidin were evaluated by HABA assay according to standard method [12].

*2.2. Protein Radiolabeling.* Radiolabeling of all proteins was performed with $^{125}I$ by IODO-GEN kit (Pierce, USA), before periodate oxidation, since iodination reaction occurs at pH 7.5 at which intermolecular formation of Schiff's bases of oxidized glycoproteins would occur. $^{125}I$-PEGavidin (hydrazone) was obtained by reacting $^{125}I$-oxidized avidin with a molar excess of 10 kDa PEG hydrazide (Nektar Therapeutics, San Carlos, USA), in 100 mM sodium acetate buffer pH 5.5, one hour at room temperature. After purification by size exclusion chromatography $^{125}I$-PEGavidin was found to contain an average of 8 PEG/avidin according to a known method [14]. ST2210 (Sigma Tau, Pomezia, Italy) was labeled with $^{111}$Indium according to standard method [13].

*2.3. Immunohistochemistry.* Avidin or OXavidin (50 $\mu$g/mouse) was injected into the muscle of one hind limb or into MDA-MB-468 human breast carcinoma xenotransplanted masses. Mice were sacrificed 48 hours after injection and treated tissues fixed and paraffin embedded. Staining of serial sections was performed with HRP-conjugated rabbit antiavidin antibody (GeneTex, Irvine, CA, USA), followed by DAB substrate.

*2.4. Animal Studies.* $^{125}I$-radiolabeled avidins (native, oxidized or OXavidin) were injected in one hind limb muscle at the dose of 50 $\mu$g in 15 $\mu$l ($6 \times 10^5$– $2 \times 10^6$ cpm/mouse). For kinetic determinations, groups of mice were sacrificed at the indicated time points and the injected sites, non target organs and blood collected and weighted and radioactivity quantified by gamma counter. For the intratissue administration, data were expressed as the % of injected dose/100 mg of tissue (% ID/100 mg) rather than % ID/g because the distribution volume of the intramuscular injection was <1 g of tissue. For $^{111}$In-ST2210 uptake mice were pretreated with OXavidin or relevant controls and then injected intravenously, at the indicated time points, with 5 $\mu$g of $^{111}$In-ST2210 ($6–20 \times 10^6$ cpm/mouse) in 100 $\mu$L of vehicle. All mice were sacrificed by $CO_2$ asphyxia 1 or 2 hours after $^{111}$In-ST2210 injection, the preavidinated site, non target organs, and blood were collected, weighted, and radioactivity quantified by gamma counter. Data were expressed as % ID/g. The care and husbandry of mice were in accordance with the European Directive 86/609 and Italian legislation.

*2.5. OXavidin Uptake of Biotinylated Cells.* Green fluorescent protein (GFP) expressing B16 melanoma cells were kindly provided by the Department of Oncology Sigma Tau SpA. Biotinylation was performed with Sulfo-NHS-LC-Biotin kit (Pierce). Chamber slides Lab-Tech II (Nange Nunc Int. Corp. IL, USA) were prepared with COS7 cell (ATCC, MD, USA) monolayer and then incubated with avidin or OXavidin (7.2 $\mu$g/mL in 100 mM sodium acetate pH 5.5) 2 hours at 4°C, washed twice with PBS, and then incubated with biotinylated or not biotinylated GFP-expressing B16 cells ($7 \times 10^5/1.8\,cm^2$), 1 hour at 4°C. After two washings with PBS, slides were evaluated by a Nikon microscope (Eclipse 80, Nikon) equipped with a digital camera DXM1200F and cell uptake was evaluated as the average of nine independent microscopic fields.

## 3. Results and Discussion

*3.1. Avidin Derivatives with Improved Tissue Half Life.* We confirmed that the tissue residence of avidin as well as that of its not glycosylated analogue streptavidin [15] is very short with less than 1% ID/100 mg present 24 hours after intramuscular injection in mice (Figure 1). Avidin, although diffusing from the injected limb as streptavidin, exhibits lower residence in blood and faster and higher liver accumulation, as expected from its known pharmacokinetic and biodistribution [11]. Streptavidin accumulates in the kidney. Therefore, in an attempt to improve the tissue half life of avidin, while reducing localization in non target organs, we conjugated it to polyethylenglycol (PEG) which is known to improve blood half life of proteins [16]. Taking into account that avidin oligosaccharides are not essential for biotin binding [8] and to minimize the possible steric hindrance of PEG on the biotin binding sites, PEGylation of avidin was performed at the carbohydrate moieties. Avidin was oxidized with sodium periodate to obtain reactive aldehyde groups (CHO) from mannose and glucosamine pyranosidic rings, according to a known method commonly used to conjugate sugars or glycoproteins to amino groups by forming Schiff's bases, or to hydrazide forming hydrazone [17]. To evaluate tissue residence, $^{125}I$-radiolabeled avidin, PEGavidin, and its reaction intermediate oxidized avidin exhibiting reactive CHO groups, all formulated at pH 5.5 to prevent intra/interprotein Schiff's base formation by oxidized avidin, were intramuscularly injected in mice. At such acidic pH, CHO groups are reactive only with hydrazino groups (pKa of hydrazide = 2.6) but are substantially inert against protein amino groups (pKa of protein amino groups = 8-9) which are in the protonated $NH_3^+$ status; at neutral and higher pH, CHO groups react with protein amino groups to form Schiff's bases. The amount of both avidin and PEGavidin, measured after 24 hours, was less than

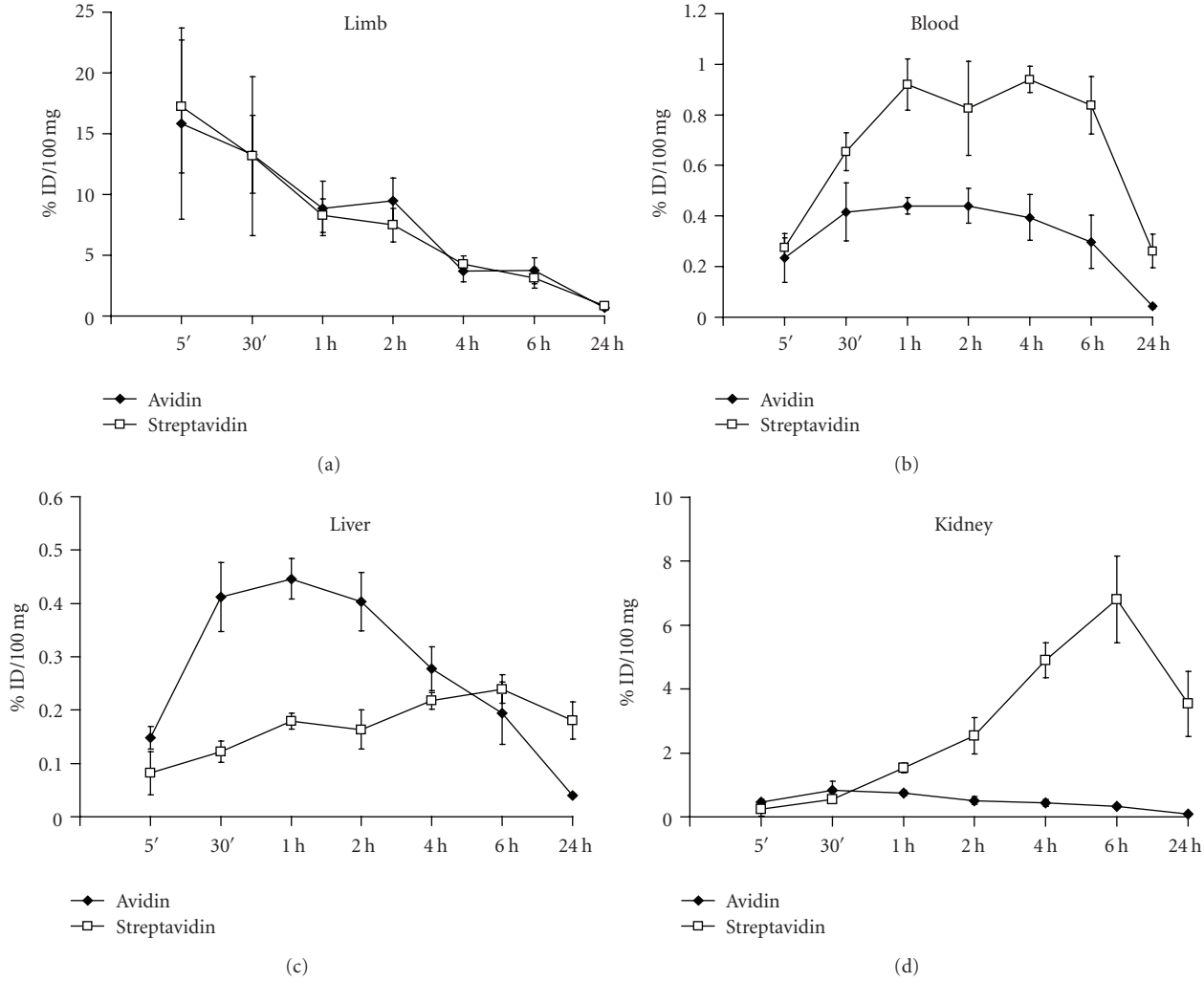


Figure 1: Diffusion kinetic of $^{125}$I-labeled avidin or streptavidin injected in a limb and distribution in blood, liver, and kidney. At indicated time points, mice were sacrificed and tissue samples weighted and counted in a gamma-counter. Data are expressed as % ID/100 mg of tissue. Each point is the average of 5 mice. Bars represent standard deviation.

1.5% ID/100 mg of tissue while, unexpectedly, the amount of oxidized avidin was about 10 times higher (Figure 2).

To further investigate this result the intramuscular residence of oxidized avidin 1, 24, and 48 hours after injection and its capacity to uptake, at the same time points, intravenously injected biotin ($^{111}$In-radiolebeled biotinDOTA ST2210) were measured. Data in Figure 3 show that, one hour after injection, the amount of oxidized avidin in the treated limb was higher than avidin and the difference increased with time. In fact, avidin was almost undetectable after 24 and 48 hours while oxidized avidin was about 17% and 14% ID/100 mg of tissue, respectively (Figure 3(a)). At the first hour, consistently with higher residence of oxidized avidin compared to avidin in the treated limb, it was observed a lower distribution of oxidized avidin in non target organs (Figures 3(b)–3(d)). The amount of both oxidized avidin and avidin in non target organs, 24 and 48 hours after their intramuscular injection, was comparable and in any case below 0.2% ID/100 mg of tissue. These kinetic data suggested that oxidized avidin could be employed for targeted administration of biotinylated agents in a time frame wider than that allowed by avidin. In fact, the uptake of $^{111}$In-ST2210, intravenously injected 24 or 48 hours after intramuscular avidin, was at least 10 times higher in oxidized avidin-treated limb than in the avidin-treated one (Figure 3(e)). Despite the higher amount of oxidized avidin in the treated limb at 1 hour, a higher uptake of $^{111}$In-ST2210 was observed in the avidin-treated one. This result was attributed to a lower biotin binding potency of oxidized avidin compared to avidin as a consequence of the expected oxidation damage of some tryptophan residues which are known to be relevant to the avidin biological activity [8]. On the other hand, at 1 hour, the distribution of $^{111}$In-ST2210 in non target organs was lower with oxidized avidin compared to avidin, particularly for kidney and liver, coherently with the fact that the higher stability of oxidized avidin at the injection site results in a reduced distribution and consequent biotin uptake in non target

Table 1: Setting up production method of OXavidin.

| | $NaIO_4$ mM | CHO/Avidin molar ratio ± SD ($N$) | ST2210 binding % ± SD ($N$) | Tissue residence % ID/100 mg ± SD 24 hours ($N$) | 1 week ($N$) |
|---|---|---|---|---|---|
| Avidin | — | <LoQ (5) | [@]100 | 2.2 ± 0.4 (7) | 0.5 ± 0.1 (5) |
| Oxidized avidin | 1 | 5.6 ± 0.8 (5) | 77.2 ± 0.9 (4) | 3.1 ± 0.7 (1) | 0.16 ± 0.03 (1) |
| | 5 | 7.5 ± 0.8 (5) | 55.7 ± 2.2 (4) | 6.3 ± 0.3 (1) | 0.99 ± 0.25 (1) |
| | 10 | 8.4 ± 1.0 (6) | 50.9 ± 2.4 (6) | 18.0 ± 2.5 (4) | 9.7 ± 3.2 (3) |
| | 20 | 11.5 ± 1.5* (7) | 49.1 ± 2.1 (6) | 19.1 ± 2.0 (4) | 11.4 ± 1.3 (3) |
| | 40 | 10.9 ± 2.6 (5) | 44.5 ± 0.9 (4) | NT | NT |
| | +HABA | | | | |
| OXavidin | 1 | 4.0 ± 0.8 (3) | 99.0 ± 0.2 (3) | NT | NT |
| | 5 | 7.1 ± 1.6 (4) | 95.0 ± 1.7 (3) | NT | NT |
| | 10 | 8.5 ± 1.3 (5) | 86.3 ± 1.6** (3) | 16.2 ± 2.3 (4) | 9.9 ± 1.3 (4) |
| | 20 | 12.9 ± 2.3[#] (6) | 81.4 ± 1.0[#]** (4) | 18.7 ± 1.1 (5) | 11.7 ± 1.7 (5) |
| | 40 | 9.8 ± 1.9 (4) | 73.0 ± 0.6 (3) | NT | NT |

$N$: Number of independent experiments; 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; *$P < .05$ versus 10 mM; [#]$P < .01$ versus 10 mM (One way Anova followed by Student-Newman-Keuls); **$P < .001$ versus same without HABA (Two-way Anova followed by Bonferroni).

organs (Figure 3(f)–3(h)). These data indicate that oxidized avidin has a longer and more specific tissue residence than avidin and suggest that oxidation might have reduced its biotin binding capacity.

*3.2. OXavidin Production and Biological Characterization.* To prevent oxidation damage of the biotin binding sites of avidin, we attempted the occupancy of the sites with the low-affinity ligand 4-hydroxyazobenzene-2′-carboxylic acid (HABA) [12], before oxidation. Several reaction conditions were tested showing that HABA-protected oxidization of avidin ($OXavidin_{HABA}$, named for simplicity OXavidin) followed by removal of HABA and formulation at pH 5.5 (to prevent intra/inter-protein Schiff's bases) allows the generation of a product that exhibits a number of CHO groups similar to oxidized avidin and positively correlating with tissue persistence measured 24 hours and 1 week after intramuscular injection. However, the biological activity of OXavidin in terms of ST2210 binding was significantly higher than that of oxidized avidin indicating that occupation of the biotin binding sites with HABA, during oxidation, is useful to preserve the protein function (Table 1). Demonstration that the persistence of OXavidin in injected tissues is due to its capacity to form Schiff's bases with tissue proteins at physiological pH was obtained by reducing aldehyde groups of OXavidin with sodium cyanoborohydride. In fact, tissue residence of such reduced OXavidin was similar to that of avidin (data not shown).

Concerning tissue localization, a much stronger and homogenous distribution of OXavidin compared to avidin, 24 hours after injection, was observed by immunohistochemistry, both in normal tissues (Figures 4(a), 4(b)) and in xenotransplanted human breast tumor masses (Figures 4(c), 4(d)). Tissue localization appeared to be in the stroma surrounding cells. We postulated that such highly efficient and homogenous tissue localization of OXavidin could be exploited to target radioactive biotin to tumors representing an appealing brachytherapy method particularly for the easiness of placement by a simple injection and for the possibility to uncouple the administration of OXavidin with that of radiolabeled biotin. In fact, based on OXavidin tissue kinetic, its administration could be performed intraoperatively/radiologically guided, followed by intravenous radioactive biotin even several days after OXavidin tissue pretargeting. This two-step brachytherapy approach could be useful in those cases where patient condition or logistic factors might otherwise discourage access to therapy.

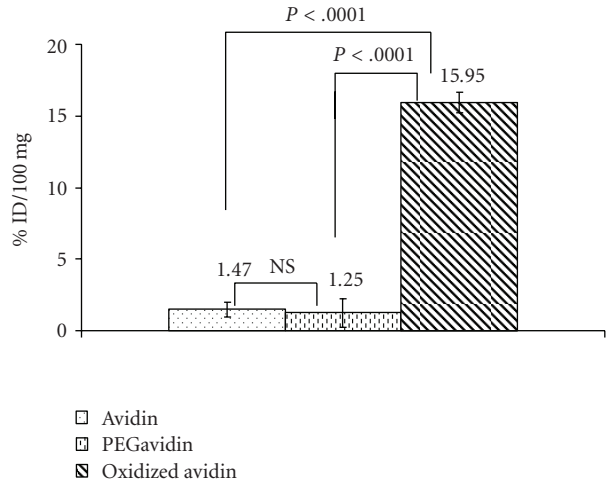


Figure 2: Tissue residence of avidin, PEGavidin, and oxidized avidin. $^{125}$I-labeled avidin, PEGavidin, and oxidized avidin were formulated in 100 mM sodium acetate pH 5.5 and injected in the limb muscle at the dose of 50 $\mu$g in 15 $\mu$L/mouse. Mice were sacrificed 24 hours after injection and tissue samples weighted and counted in a gamma-counter. Data are expressed as % ID/100 mg of tissue. Each point is the average of 5 mice. Bars represent standard deviation. Student's $t$ test was used for statistical analysis. NS: Not significant.

Therefore, a mouse model was performed as a proof of principle of such pretargeted brachytherapy. Mice were injected in one hind limb with either avidin or OXavidin and after 48 hours received intravenous $^{111}$In-ST2210. The mice were sacrificed at the indicated time points and radioactivity in the treated limb as well as in other non target organs measured by gamma counter. The amount of $^{111}$In-ST2210 after 2 hours from intravenous injection was about 150 times higher in the OXavidin-treated tissue than in the avidin-treated one (Figure 5(a)). Significant difference in radioactivity level persisted at the subsequent time points up to 24 hours from $^{111}$In-ST2210 intravenous injection when about 63 times more radioactivity was found in the tissue treated with OXavidin compared to avidin. The distribution of $^{111}$In-ST2210 in non target organs was similar for avidin and OXavidin and at all time points below 0.2% ID/g of spleen and liver and below 2.0% for kidney (Figures 5(b), 5(c), 5(d), resp.). The area under the curve (AUC) of $^{111}$In-ST2210 distribution into the treated limb resulted about 100 times that of avidin while AUCs of avidin and OXavidin in non target organs were very similar. Present data confirm that OXavidin stably binds tissues and efficiently uptakes radioactive biotin thus supporting its possible use in an innovative form of pretargeted brachytherapy with radiolabeled biotin like $^{90}$Y-ST2210.

For clinical applications where a delayed administration of the biotinylated therapeutic is not necessary, the use of the OXavidin/biotinylated therapeutic preformed complex is also possible. As shown in Figure 6, 24 hours after intramuscular injection, the avidin-biotinDOTA and OXavidin-biotinDOTA complexes were about 5% and 24% ID/100 mg of tissue, respectively. The amount of the avidin-biotinDOTA complex was about twice that of avidin and this statistically significant difference was expected as it was previously described that the biotin engagement induces conformational modification of avidin increasing its tissue residence most likely by enhancing sugar exposition and nonspecific interaction [10]. This mechanism is unlikely to occur for OXavidin-biotinDOTA complex as the avidin oxidation replaces sugars with aldehyde groups responsible for tissue binding of both OXavidin and OXavidin-biotinDOTA complex. One day after intramuscular injection, slightly higher binding of OXavidin compared to the OXavidin-biotinDOTA complex was observed. One week after injection, both avidin and avidin-biotinDOTA complex were below 0.5% ID/100 mg of tissue while the amount of both OXavidin and OXavidin-biotinDOTA complex was about 17% ID/100 mg. It is to note that such highly efficient tissue delivery and residence of the OXavidin-biotinDOTA complex would result in a very low amount of radioactivity needed to deliver a biological effective dose to a tumor that would translate into significant cost reduction of brachytherapy and improved compliance of both hospital personnel and patients.

The use of OXavidin for brachytherapy will offer a number of advantages compared to current brachytherapy devices. In fact, the perfusion of a target tissue with OXavidin, compared to current devices, will allow to conform more easily the therapy to the tumor/organ shape, will result in a more homogeneous interstitial distribution of the therapeutic agent, and will allow to delay of several days the administration of biotinylated therapeutics which might be also fractionated in repeated doses. As OXavidin stably links tissue proteins there will be no need to immobilize the treated tissue as needed in current seeds brachytherapy, to prevent migration of the seeds. Moreover, as most of the injected OXavidin binds and resides within the treated tissue, it will not be necessary to perform a "chasing step" with biotinylated albumin to block biotin uptake in non target organs as done in IART® [6].

In order to evaluate the possible use of OXavidin to uptake biotinylated agents larger in size than biotinDOTA, avidin or OXavidin was added to a monolayer of COS7 cells grown on chamber slides and, after washings, the slides were incubated with GFP-expressing B16 melanoma cells both biotinylated and not biotinylated. Figure 7 shows that $1.25 \pm 1.14$ and $1.42 \pm 1.16$ B16 cells/field were bound to avidin- and OXavidin-treated COS7 slides, respectively. On the other hand, $4.17 \pm 2.44$ and $45.75 \pm 1.39$ biotinylated B16 cells/field were counted on avidin- and OXavidin-treated COS7 slides, respectively. Overall, data indicate that this cell-cell binding is OXavidin/biotin mediated and suggest the possible use of OXavidin to uptake biotinylated cells in treated tissues.

The use of an active, tissue binding compound like OXavidin which, to our knowledge, has no precedent example in pharmaceutical drugs might raise safety concerns. However, it should be taken into consideration that present in vivo studies indicate good tolerability as neither systemic clinical signs nor histological lesions were observed in the animals

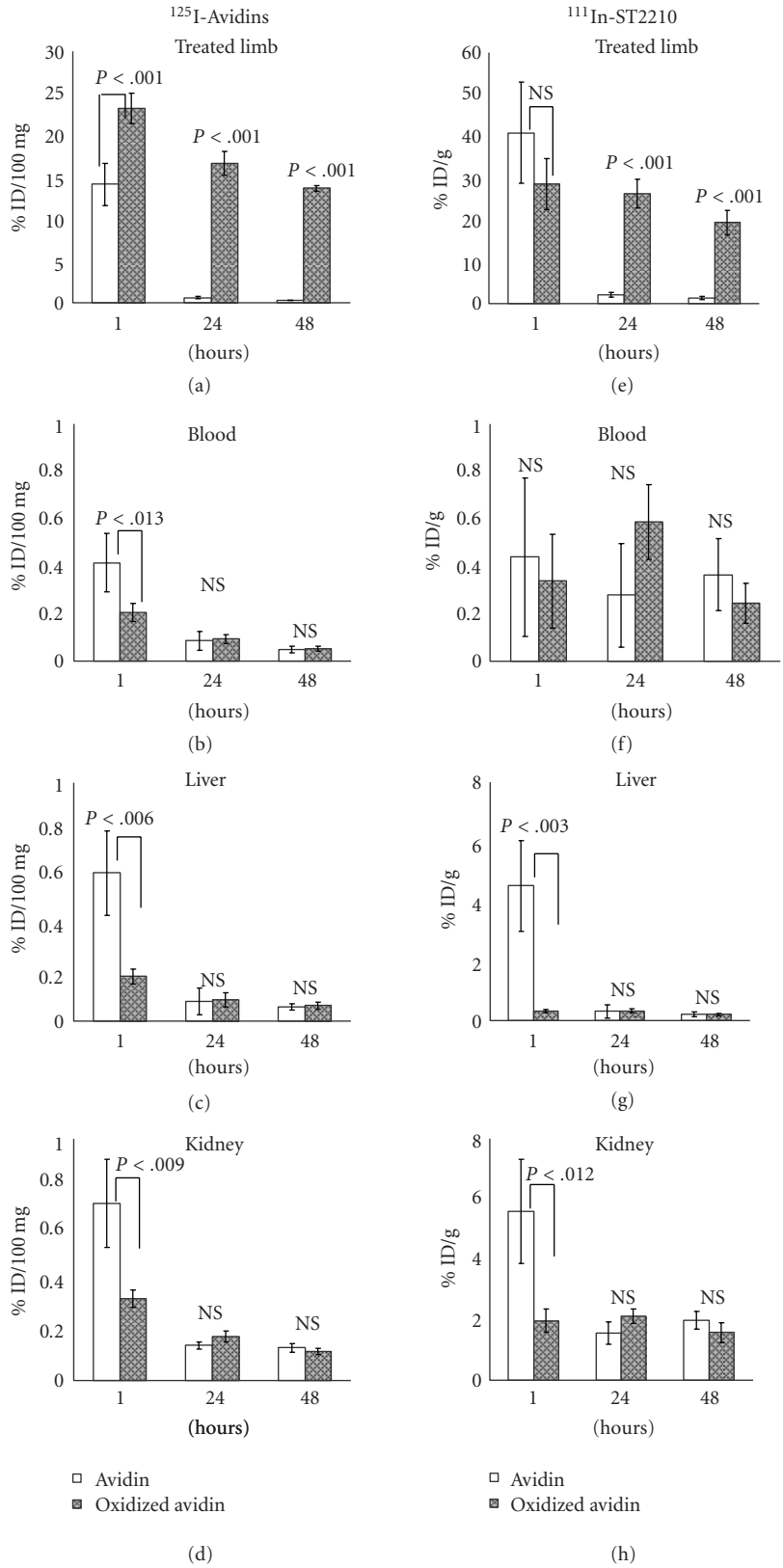


Figure 3: Oxidized avidin in vivo residence and biotin uptake. Diffusion kinetic of $^{125}$I-labeled avidin and oxidized avidin injected in a limb of Balb/c mice (a)–(d) and uptake of intravenously administered $^{111}$In-ST2210 (e)–(h), after 1, 24, or 48 hours. Mice were sacrificed 1 hour after $^{111}$In-ST2210 injection and injected sites as well as samples of non target organs were weighted and counted in a gamma-counter. Each point is the average of 5 mice. Bars represent standard deviation. Student's $t$ test was used for statistical analysis. NS: Not significant.

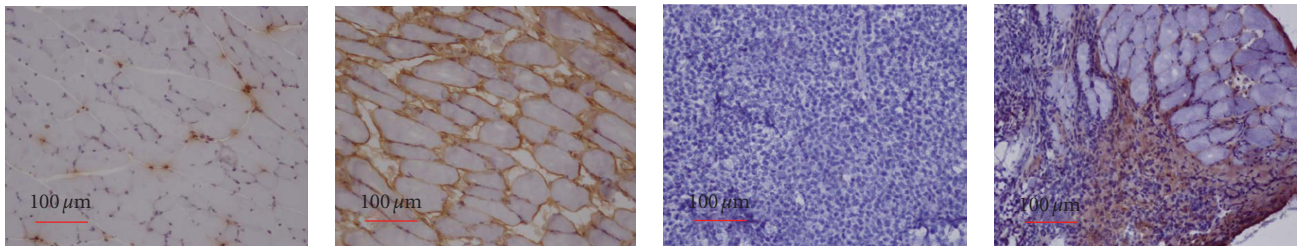


Figure 4: Immunohistochemistry of avidin (a,c) or OXavidin (b,d) injected normal muscle (a,b) or MDA-MB-468 human breast carcinoma xenotransplanted mass (c,d) 48 hours after injection.

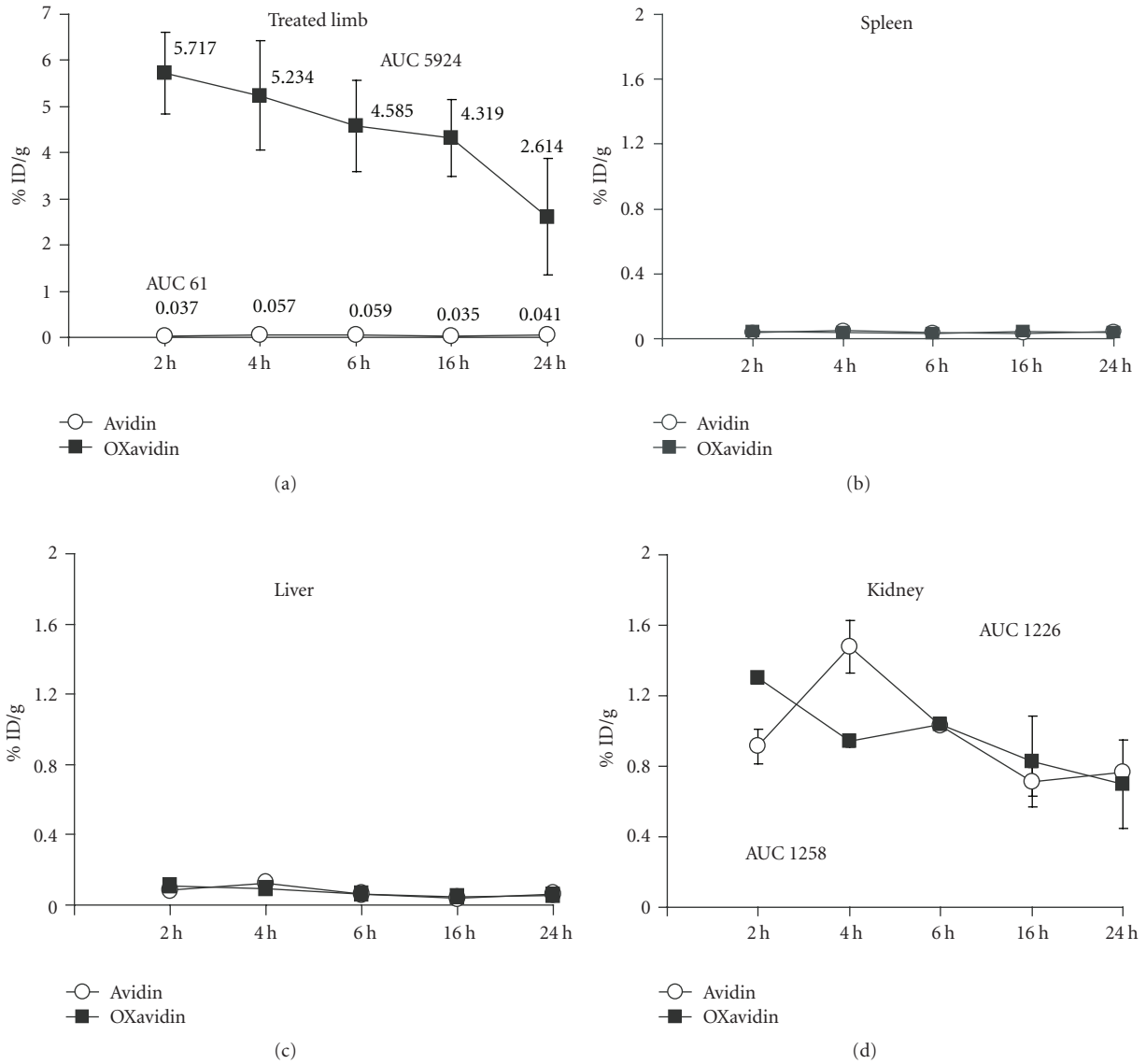


Figure 5: Uptake of $^{111}$In-ST2210, intravenously injected 48 hours after intramuscular administration of OXavidin or avidin in a limb. Groups of mice were sacrificed at the indicated time points after $^{111}$In-ST2210 injection and samples of treated limb, spleen, liver, and kidney weighted and counted in a gamma counter. Data are expressed as % ID/g of tissue. Each point is the average of 5 mice. Bars represent standard deviation.

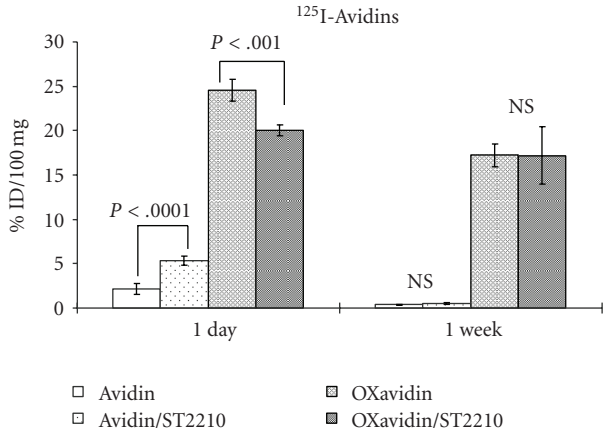


Figure 6: Tissue residence of OXavidin/biotin complex. $^{125}$I-labeled avidin, avidin/ST2210, OXavidin, or OXavidin/ST2210 were injected in the limb of Balb/c mice. At the indicated time points after intramuscular injection mice were sacrificed and samples of treated limb weighted and counted in a gamma counter. Data are expressed as % ID/100 mg of tissue. Each point is the average of 5 mice. Bars represent standard deviation. Student's *t* test was used for statistical analysis. NS: Not significant.

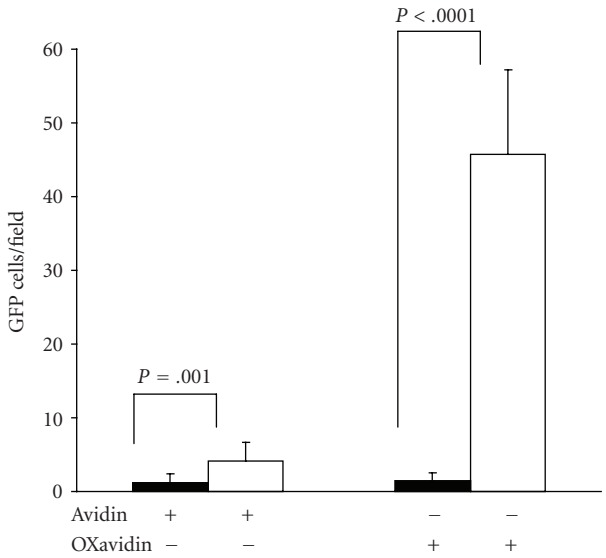


Figure 7: OXavidin uptake of biotinylated cells. OXavidin shows highly efficient uptake of biotinylated GFP-expressing B16 cells compared to avidin. Black and white histograms indicate the average of nine fields of B16 and biotinylated B16 cells, respectively. Bars represent standard deviation. Student's *t* test was used for statistical analysis.

treated with OXavidin. Moreover, the formation of Schiff's bases between oxidized sugars and protein amino groups is a common event in vivo, leading to protein glycation [18] and finally it is known that hundreds of oxidized proteins are physiologically produced in the body as common products of oxidative stress [19]. We believe that it will be possible to employ OXavidin for highly efficient and specific tissue targeting of therapeutic agents like radioisotopes, drugs, growth factors, plasmids, viral vectors, or cells for each of which biotinylation protocols are available [20–22].

## 4. Conclusion

In summary, a novel reagent is described named OXavidin that preserves the biotin binding capacity of avidin while exhibiting the property to chemically link tissues. This product is obtained by HABA-assisted oxidation of avidin. The production method is very convenient because, in a perspective of OXavidin industrial development, HABA is a nontoxic ligand and, for its low affinity, it can be easily removed after oxidation. Overall in vitro and in vivo data suggest that OXavidin has a remarkable potential for a variety of therapeutic applications.

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