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Albumin-oxanorbornadiene conjugates formed *ex vivo* for the extended circulation of hydrophilic cargo

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Abstract

Oxanorbornadiene dicarboxylate (OND) reagents were explored for the purpose of binding and releasing chemical cargos from endogenous circulating serum albumins. ONDs bearing gadolinium chelates as model cargos exhibited variable conjugation efficiencies with albumin in rat subjects that are consistent with the observed reactivity of each linker and their observed stability toward serum hydrolases *in vitro*. The terminal elimination rate from circulation was dependent on the identity of the OND used, and increased circulation time of gadolinium cargo was achieved for linkers bearing electrophilic fragments designed to react with cysteine-34 of circulating serum albumin. This binding of and release from endogenous albumin highlights the potential of OND linkers in the context of optimizing the pharmacokinetic parameters of drugs or diagnostic agents.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

Methods, chemical synthesis, full experimental procedures, supplemental kinetic experiments with fluorogenic ONDs, pharmacokinetic plots, and spectral characterization of new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

Albumin is an abundant and versatile endogenous plasma protein that has gained popularity as a carrier for improving the pharmacokinetic profiles of protein- and peptide-based drugs, as well as drugs with suboptimal circulation lifetimes.^{1,2} Human serum albumin has a relatively long half-life in blood of 19 days and plays an important role as a binder, carrier, and solubilizing agent for long chain fatty acids, various metal ions, exogenous drugs, and bilirubin, the toxic metabolic breakdown product of heme.³ Additionally, albumin accumulates in solid tumors and sites of chronic inflammation, such as in rheumatoid arthritis, and can serve as a source of nutrients and amino acids in peripheral tissues.^{1,3–5} Albumin itself is non-toxic and non-immunogenic and is very soluble, as evidenced by its high concentration in plasma.⁶ Finally, mammalian albumins display an abundance of solvent-exposed amine nucleophiles, and contain a conserved free cysteine (cys-34). This cysteine serves important roles in the binding of metals, and in the transport of nitric oxide in the blood. The free thiol form of the protein, mercaptalbumin, accounts for 90% of extracellular (plasma) thiols in mammals, presenting a unique opportunity for the use of thiol-modifying agents to form bioconjugates.^{3,7–9}

These characteristics have made albumin an attractive carrier for the design of macromolecular prodrugs and preclinical diagnostic agents. Three main approaches have been explored in this regard: (1) the covalent or non-covalent binding of small molecule pharmacophores to exogenous or endogenous albumin, (2) the synthesis of albumin-based nanoparticle therapeutics, and (3) the fusion of bioactive proteins to circulating albumin. A survey of efforts in these areas has been presented in recent reviews.^{1,2} Here we describe a variant of the first of the above strategies, focusing on the covalent attachment of thiol-reactive oxanorbornadiene (OND) dicarboxylate linkers to endogenous circulating albumin in rodent models as a method of tuning the pharmacokinetic profile of hydrophilic molecular cargos.

ONDs are potent electrophiles that undergo Michael addition with thiols and amines to form adducts that subsequently fragment by retro-Diels-Alder reaction. The rate of fragmentation varies with the identity of the parent OND, and can be tuned over a wide range under physiological conditions.^{10,11} Fragmentation-resistant epoxyoxanorbornene (EONB) derivatives can be prepared by epoxidation of OND linkers at the electron-rich alkene.^{10,12} We have previously described the use of this linker technology to achieve conjugation and release of fluorescent cargo from bovine and human serum albumins, with release half-lives ranging from 40 minutes to nearly 1 week under simulated physiological conditions.¹¹ We

describe herein the first application of this chemistry *in vivo* to modulate the pharmacokinetic parameters of gadolinium chelators as model hydrophilic cargos.

One of the first albumin-drug conjugates tested in phase I and II clinical trials was the methotrexate-human serum albumin (MTX-HSA) conjugate, investigated for treatment of cancers and rheumatoid arthritis as a potential replacement for free methotrexate, which suffers from poor selectivity and suboptimal pharmacokinetics.^{4,13–20} The material was prepared by conjugation of a methotrexate derivative to the pendant lysine sidechains on HSA *in vitro* (Figure 1a), an approach not without its drawbacks. The MTX-HSA conjugate was not chemically defined due to low specificity of labeling, and its pharmacokinetic and clinical performance depended strongly on the number of molecules conjugated to the albumin carrier, with an optimal loading of approximately one MTX per molecule of albumin.¹⁶ Additionally, the prodrug nature of MTX-HSA has not been clearly established; the rates of cleavage of MTX cargo from its protein carrier and the identity of these cleavage products have not been determined.

With these problems in mind, Kratz and colleagues designed Aldoxorubicin[®], a doxorubicin prodrug targeted to cysteine-34 of endogenous albumin.^{9,21,22} Upon injection, this drug rapidly reacts with mercaptalbumin to form well-defined circulating depots (Figure 1b).²¹ The prodrug contains an active doxorubicin pharmacophore conjugated to a thiol-reactive maleimide through an acid sensitive hydrazone linkage. The design was derived from a need to control conjugation site and the stoichiometry between drug and carrier, and to modulate drug accumulation and release site. In this case, release is achieved by accelerated cleavage of the acid-sensitive hydrazone linkage in the low pH environment of solid tumors.²³ This prodrug has achieved preclinical success, and has advanced to Phase III clinical trials.^{24,25} The strategy of incorporating an albumin-targeted electrophile and a cleavable linker offers a few key advantages over conjugates comparable to MTX-HSA. By targeting endogenous circulating albumin, the need to form conjugates *in vitro* using commercially available albumins are avoided. Additionally, the chemical nature of the prodrug can be established almost as easily as any other small molecule drug. The use of a cleavable linker also offers improved control over cleavage products and release kinetics.

The design of our OND-cargo materials is similar to Aldoxorubicin[®] in that we incorporate both an attachment and release function, as well as a modular design for conjugation of a wide range of chemical cargos (Figure 1c). A key difference is that ONDs provide temporal control over release rate, and their modularity allows for adjustment of that rate; the stimulus responsive release of hydrazone linkages are more difficult to adjust.^{10–12} Our design is complementary to recently developed synthetic macromolecular prodrugs employing β -eliminative linkers, in which a wide range of release rates under physiological conditions allows fine-tuning of cargo pharmacokinetics.^{26,27} However, our design potentially circumvents the need of preparing macromolecular conjugates *in vitro* and cleavage of OND adducts occurs by a pH insensitive mechanism.¹⁰ This binding and release of endogenous long-circulating carriers may prove useful in the context of optimizing the pharmacokinetics of drug molecules or diagnostic agents.

RESULTS AND DISCUSSION

Design and Synthesis of Gd-functionalized ONDs

Gadolinium-bearing ONDs were prepared based on the coordinating ligand DOTA.²⁸ In this case, the cyclen derivative DO3A was employed, in which one of the four acetate groups is differentially substituted to facilitate attachment of a functional moiety, such as an OND derivative, maleimide, or furan (compounds **1–4**, Figure 2). We designed these molecules not for their potential use as contrast agents in ¹H-MRI imaging, but rather to facilitate detection and quantification in whole blood by inductively coupled plasma atomic emission spectroscopy (ICP-AES).^{29,30} Additionally, the pharmacokinetic behavior of similar gadolinium complexes is well characterized in animal models.²⁸ We selected two different ONDs with widely differing adduct stabilities, and compared these to the “non-cleavable” EONB and maleimide derivatives.^{11,12} Furan **4**, the cargo released from OND-nucleophile adducts upon retro-Diels-Alder (rDA) fragmentation, and itself incapable of conjugating to serum albumin, was prepared as a control. Based on previous work with [Gd•DOTA] in rats, it was expected that **4** would have a very short circulation lifetime ($t_{1/2}$ ~20 minutes).²⁸

These compounds were prepared by N-alkylation of a tris(*tert*-butyl ester) DOTA amine with bromoacetamide derivatives of each electrophile, followed by deprotection and Gd(III) insertion (Supporting Information). Maleimide **3** was protected as the Diels-Alder adduct with furan to avoid maleimide hydrolysis during gadolinium insertion. Unexpectedly, thermal deprotection of this adduct by retro-Diels-Alder reaction was sluggish in toluene alone, but proceeded smoothly in the presence of 2% v/v DMSO co-solvent (Scheme S1B). Gadolinium-bearing species were purified by semi-preparative HPLC prior to evaluation of *in vitro* reactivity with isolated rat serum albumin.

Reactions with Serum Albumin Preparations

Bioconjugation reactions were performed in the presence of both thiol-rich and thiol-depleted rat serum albumin (RSA), the latter prepared by saturating free thiols with N-ethyl maleimide. The concentrations of RSA and electrophiles in these experiments were selected to approximate the pre-incubation and injection conditions employed in the stages of *in vivo* circulation experiments (see below). First, the concentration of RSA was held constant at 20 mg/mL (310 μ M), roughly the concentration of RSA in blood, and the electrophile was present at sub-stoichiometric concentration (143 μ M) relative to albumin. After 10 minutes of reaction at 37 °C, reactions were quenched by passage through size-exclusion resin. Collected fractions were analyzed both for protein content and gadolinium concentration to generate the fractionation profiles in Figures 3 and 4. In all cases, RSA eluted in the first 2 mL, while the elution volume of each Gd chelate varied with linker identity and extent of labeling.

Controls **3** and **4** performed as expected, as shown in Figure 3E and 4E, respectively: RSA and Gd content eluted in two separate peaks in the chromatogram of RSA+**4**, while almost perfect co-elution was observed for linker **3**, consistent with nearly complete labeling of RSA with the maleimide but no labeling with the furan. Linker **1b** performed similarly to maleimide **3** (Figure 3C), but compounds **1a** and **2** proved to be less reactive, giving

intermediate levels of labeling as indicated by the appearance of a portion of Gd-chelate eluting separately from the protein fraction (Figure 3B,D).

In order to determine the extent to which the primary amine groups of RSA play a role in protein labeling, **1a**, **1b**, and **3** were incubated with RSA that had been pre-treated with N-ethylmaleimide to cap the cysteine-34 thiol (Figure 4). Compounds **1a** and **3** performed similarly, undergoing significantly less conjugation with thiol-capped RSA than with untreated (reduced) protein, consistent with previous observations that OND dicarboxylic esters closely resemble maleimides in chemoselectivity for thiols vs. amines.^{11,12} In contrast, trifluoromethyl OND **1b** proved to be much more reactive with protein amino groups, showing nearly complete attachment to both maleimide-treated and untreated RSA. In a separate experiment, the reaction of a roughly ten-fold excess of **1a** (3.5 mM) with RSA (310 μ M) at room temperature (23 °C) for 15 minutes gave an adduct mixture with a 1.2:1 ratio of Gd to protein, by quantitative analysis of the resulting protein (Figure S3). The identical reaction using **1b** resulted in a much greater degree of labeling (Gd:protein $\approx$ 3.7:1), while the same reaction using **4** resulted in no labeling.

Further studies of the chemoselectivity of fluorogenic OND electrophiles similar to **1a**, **1b**, and **2** are described in Supporting Information (Figures S4–S6). Reactions were performed with bovine and rat serum albumins, as well as N-acetylcysteine and aminocaproic acid as representative thiol and amine nucleophiles. In all cases, a similar selectivity trend was observed, in which trifluoromethyl/carboxyester derivatives reacted with thiol-depleted bovine albumins and aminocaproic acid faster than dicarboxyester and EONB compounds. No appreciable difference in thiol reactivity was observed between these various electrophiles in condensations with N-acetylcysteine, showing that the more promiscuous behavior of compounds like **1b** is due to enhanced amine reactivity and not diminished rates of thiol addition. Interestingly, reactions with rat serum albumin were faster by 7 to 14 fold than with bovine serum albumin for each electrophile tested, depending on the reagent (Figure S5).

The *in vitro* release behavior of RSA adducts of **1a**, **1b**, and **3** was examined by incubating Gd-tagged RSA at 37 °C, analyzing aliquots for Gd and protein content as above. A steady decline in [Gd] associated with the protein fraction was observed in samples containing RSA treated with **1a** (Figure 5A). In contrast, Gd levels in samples of RSA treated with either **1b** or **3** were relatively stable over prolonged incubation. This behavior is consistent with the relative stabilities of model thiol- and amine adducts of these electrophiles observed previously.^{11,12} The decrease in [Gd] with time for linker **1a** does not fit a first-order model (a plot of ln[Gd] vs. time is decidedly curved, not shown), indicating that more than one release process occurs at the same time. Instead, the observed release behavior of RSA-**1a** adducts was nicely modeled as two simultaneous first-order release processes (retro-Diels-Alder fragmentation of RSA-**1a** thioland amine-adducts respectively, Figure 5B), as previously characterized for fluorogenic OND-dicarboxylate adducts of bovine serum albumin.¹¹ The optimized fit provided half-lives of 9.0 hours and 13 days for the fragmentation of RSA-**1a** thiol- and amine-adducts, respectively, and mole fractions of approximately 0.75 for thiol adducts and 0.25 for amine adducts. All of these values are consistent with our prior experience with analogous small-molecule adducts.

Assessment of OND Stability in Plasma

The stability of OND electrophiles toward serum hydrolases was examined using model compounds **5**–**9** (Figure 6), representing the classes of linkers examined *in vivo* (see below). These compounds were incubated in rat plasma at a sufficiently high concentration (1 mM) to completely deplete albumin thiols, and their concentration was monitored by mass spectrometry. Methyl esters **5** and **7** were found to be much less stable under these conditions than ethyl ester **6**, whereas maleimide **8** and furan **9** were largely unaffected. The conclusion that esterases rather than amide hydrolases were primarily responsible for the rapid loss of **5** and **7** was supported by the observation of increasing amounts over time of the expected carboxylic acid hydrolysis products rather than amines (see Supporting Information for details, Figures S9–S13). Heat inactivation of plasma hydrolases by pre-heating of the serum at 57 °C greatly increased the stability of **5** and **7**, but had little effect on the stability of **6** (Supporting Information, Figure S8, Table S2). Thus, the loss of **6** is probably not predominantly an enzymatic process, but rather represents slow reaction of the trifluoromethylated electrophile with protein amine groups. OND carboxylic acids exhibit significantly slower rates of Michael addition, which would be expected to lower bioconjugation efficiency *in vivo*.^{12,31}

Pharmacokinetics of Gd-ONDs in Rats

Conjugation of Gd-OND reagents to serum albumin *in situ* was tested as an approach to increased serum residency for hydrophilic cargos that exhibit rapid elimination from the blood. [Gd•DOTA] has a reported half-life of roughly 19 minutes in adult rats.²⁸ Reactive linkers **1b**, **2**, and **3** should allow for rapid attachment to rat serum albumin (RSA), and therefore for retention of Gd in the blood. We expected to observe profiles approximating that of RSA itself, since adducts of these electrophiles have rDA half-lives (>20 days for thiol adducts of **1b** analogs) that far exceed the clearance half-life of RSA in the rat (approx. 48 hours; see Supporting Information).¹⁷

Preliminary experiments tested the injection of saline solutions of **1a**, **2**, or **4** over 1 minute in adult male Sprague-Dawley rats, but this protocol provided lower than expected concentrations of gadolinium in the blood (data not shown). This was attributed to incomplete bioconjugation reaction prior to distribution and elimination of unconjugated electrophiles, the sluggish reactivity caused in part by rapid ester hydrolysis. We therefore used a modified injection protocol to enhance RSA conjugation efficiency. At the start of each experiment, 1 mL of blood was withdrawn into a syringe containing heparin and the Gd-bearing test compound at a dose of 10 $\mu\text{mol/kg}$ and rotated at room temperature for 15 minutes prior to reinjection. The concentration of test compounds in this phase was approximately 3.5 mM, and was expected to allow conjugation to compete somewhat with the action of plasma esterases. After injection (defining the t_0 time point), blood samples were periodically withdrawn, stored, and were later digested in concentrated nitric acid with the aid of a microwave reactor to provide homogeneous digests amenable to analysis by ICP-AES (see Supporting Information, Figure S14).

The results, in terms of observed gadolinium concentrations in serum *vs.* time, are presented in semi-log format in Figure 7. As expected, the furan **4** was rapidly eliminated from

circulation with a terminal half-life of 20 minutes (Table 1), consistent with this linker being unreactive toward carrier protein nucleophiles. In contrast, compounds **1b** and **3** exhibited almost identical behavior, with a non-linear distribution phase followed by a linear terminal elimination phase. Relatively long terminal half-lives (33.8 hours for **1b**, and 30.3 hours for **3**, respectively) were observed. The prolonged circulation of cargo attached via these OND linkers is consistent with their high relative stability toward retro-Diels-Alder fragmentation, and is nearly identical to the elimination behavior reported for both radiolabeled RSA and methotrexate-RSA lysine conjugates.¹⁷ This suggests that these conjugates are eliminated at a rate that is comparable to the rate of RSA turnover.

Unique among these self-cleaving adducts was **1a**, which, as expected, was eliminated from circulation at a rate between the non-cleavable and non-conjugated control molecules, and very closely matching the fragmentation rate of RSA-**1a** thiol adducts (Figure 5B), suggesting that OND linker cleavage can mediate the release of cargo from the albumin carrier, and therefore control its circulation lifetime.

However, quantitative analysis of the serum samples showed significantly lower initial [Gd] concentrations in serum for linker **1a** compared to derivatives **1b** and **3**, suggesting that less of the first compound was connected to RSA before systemic injection. While **1a** was less reactive *in vitro* than **3**, the difference in systemic circulation was of significantly greater magnitude. In addition, the elimination rate of epoxy derivative **2** ($t_{1/2} = 1.07 \pm 0.39$ h) was comparable to that of the non-conjugating furan **4** (Figure 7) even though its *in vitro* electrophilic reactivity was similar to **1a**. These differences suggest the presence of a competing process leading to electrophile deactivation in blood, preventing efficient labeling. The most likely such transformation is hydrolysis of either ester group of **1a** and **2**; ring opening of the epoxide group of **2** would also have the same effect. However, enough **1a** did make its way onto the RSA thiol to establish rDA cleavage as a feasible way to alter the clearance profile of the small-molecule cargo.

Sites of Adduct Formation

The expected rapid reactivity of linkers **1a**, **1b**, and **3** with peptidic thiol was verified by reaction with the model peptide CSYDEHAK, corresponding to amino acids 58–65 of mouse serum albumin (Supporting Information, Table S3). This was expanded to evaluate the reaction with full-length rat albumin, which has the potential to be modified at a number of sites other than Cys-34, since all of the electrophiles, and especially **1b**, can be expected to accept amine nucleophiles as well. To determine the most common sites of modification of RSA upon injection, blood was isolated from rats shortly after injection of **1a**, **1b**, and **3**. Each sample was digested with lys-c/trypsin and analyzed by HPLC-MS/MS. The resulting spectra were matched to a merged UniProtKB/SwissProt fragment database and searched with PEAKS software (version 7.5).³² This search revealed the peptide containing cysteine-34 (CPYEEHIK) to be the dominant site of labeling by **1a**, **1b**, and **3**; no modifications of RSA were observed with non-conjugating furan **4**.

It should be noted that peptide adducts with intact **1a** were not observed. Rather, the observed derivative was the peptide-thiomaleate derivative resulting from rDA fragmentation

of the thiol-OND adduct. This is consistent with the known rapid nature of cycloreversion of similar adducts, particularly under mass spectrometry conditions.^{12,33} Conversely, intact RSA-**1b** conjugates at Cys-34 were observed in abundance, highlighting the greater stability of these adducts.¹¹ Adducts of **3** with Cys-34 were identified as the hydrolyzed succinimide (Supporting Information).

While Cys-34 was the major site of modification, a number of additional amino acid residues of RSA were also found to be modified by **1a**, **1b**, and **3** in these studies. Four lysine adducts with OND diester **1a** were characterized (K212, K236, K460, and K490), whereas EOND **1b** was much more promiscuous, forming identifiable adducts at eight off-target sites, including five lysines (K236, K421, K499, K549, and K588), two arginines (R169, R361), and one glutamic acid (E529). A number of unidentified residues on RSA were also modified by **3**, evident by the appearance of the gadolinium isotope pattern in mass spectra of unidentified peptides in plasma digests. These results are summarized in Table S3 in Supporting Information.

CONCLUSIONS

Within the limits imposed by the relatively short half-life of rat serum albumin in rats, we have demonstrated that OND electrophiles can react rapidly with albumin Cys-34 upon exposure to blood, and that this event can be used to tailor the circulation lifetime of hydrophilic cargos *in vivo* by virtue of a programmable retro-Diels-Alder cleavage event. We also identified several off-target sites of RSA modification by ONDs **1a** and especially trifluoromethylated **1b**, increasing the fraction of cargo that remains conjugated to circulating proteins, but diminishing control over the labeling site. This highlights the need to be aware that any electrophile, including those commonly assumed to be selective for thiols, can react at multiple sites. Connection to amines and the location of off-target attachment should be assessed on a case-by-case basis when evaluating protein-derived macromolecular carriers using most common labeling methodologies.

The observed serum hydrolytic lability of OND methyl esters is a concern for applications requiring rapid labeling in plasma, or prolonged circulation of an active OND electrophile, such as in thiol-triggered drug release systems. We are exploring other ester and electron-withdrawing derivatives to overcome this challenge. Because of their modular and tailorable nature, however, ONDs remain promising linkers for modulation of pharmacokinetic behavior of a wide range of cargos by attachment to long-circulating macromolecular carriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Previous strategies employing albumin as a drug carrier, and the strategy explored in this work. (A) Methotrexate-HSA formed *in vitro* by amide coupling; (B) Aldoxorubicin[®] takes advantage of maleimide reactivity to form albumin conjugates *in vivo*, with cargo release achieved by acid-mediated hydrazone hydrolysis; (C) OND electrophiles are expected to react with circulating albumin, furnishing adducts that fragment with a tunable rate, varying with the identity of the parent OND electrophile, and provide a means of extending circulation and fine-tuning the kinetic behavior of various cargos.

Figure 2.
Gadolinium•DO3A derivatives 1–4.

Figure 3.

In vitro labeling of rat serum albumin under simulated physiological conditions. (A) Reaction of electrophilic species **1–3** with rat serum albumin; (B–E) Size exclusion chromatograms after 10-minute incubation of electrophiles **1–3** with rat serum albumin. In each fraction, [Gd] was determined by ICP-AES, and [RSA] was determined by Abs₂₈₀ measurements against a calibration standard.

Figure 4.

(A–D) *In vitro* labeling of thiol-depleted RSA, under the same conditions described in Figure 3. (E) Incubation of RSA containing thiols in the presence of furan **4** results in no labeling of the protein.

Figure 5.

(A) [Gd] co-eluting with protein fraction (2 mL elution volume) in RSA labeled with **1a**, **1b**, and **3**. (B) Fit of **1a** release profile (black line) to two simultaneous first order release processes, shown individually as dashed red and dotted blue curves.

Figure 6. Stability of selected model compounds in plasma at 1 mM, representing the major classes of linkers examined *in vivo*. The red lines on the structures at the top indicate potential sites of hydrolysis by plasma hydrolases.

Figure 7. Semi-log plot of observed gadolinium concentration in acid-digested whole blood samples collected from rats treated with compounds **1–4**, determined by ICP-AES. Error bars represent standard deviation in concentration, N = 4 rats.

Table 1

Observed Elimination Rates and Half-lives.

Linker	k_{elim} (h ⁻¹) ^a	$t_{1/2}$ (h) ^b
1a	0.077 ± 0.007	8.96 ± 0.09
1b	0.020 ± 0.002	33.8 ± 0.1
2^c	0.643 ± 0.247	1.07 ± 0.39
3	0.023 ± 0.003	30.3 ± 0.1
4^c	2.08 ± 0.18	0.333 ± 0.087

^(a) Observed elimination rate constant, determined from terminal (linear) portion of semi-natural log concentration vs. time curves of data shown in Figure 5.

^(b) Calculated from $\ln(2)/k_{\text{elim}}$.

^c Calculated using all data points, due to rapid elimination.