Protein Binding

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# A Portable Albumin Binder from a DNA-Encoded Chemical Library**

*Christoph E. Dumelin, Sabrina Trüssel, Fabian Buller, Eveline Trachsel, Frank Bootz, Yixin Zhang, Luca Mannocci, Susanne C. Beck, Mihaela Drumea-Mirancea, Mathias W. Seeliger, Christof Baltes, Thomas Müggler, Felicitas Kranz, Markus Rudin, Samu Melkko, Jörg Scheuermann, and Dario Neri**

Albumin represents the most abundant protein in human plasma, at a concentration of 45 mg mL$^{-1}$. To keep physiological production rates to a minimum, albumin displays a long circulatory half-life in mammals thanks to its size above the renal filtration threshold and its unique ability to interact with the neonatal FcRn receptor.[1] Fusions of biopharmaceuticals to albumin[2] or to albumin-binding peptides[3,4] have been devised to expose the body to adequate concentrations of the therapeutic agent for a sufficiently long period of time, thus improving efficacy and reducing the number of injections.

In principle, small organic albumin-binding molecules could be used as functional analogues of albumin-binding peptides. However, although many small molecules are known to bind to albumin, the success in isolating small molecules as portable albumin-binding moieties has been limited,[5] mainly because most albumin binders (for example, ibuprofen) lose binding affinity upon chemical modification. Myristoylation of insulin has been shown to significantly prolong the circulatory half-life,[6] but this modification is not applicable to a broader set of molecules because of its negative effect on solubility. In another example, a 4,4-diphenylcyclohexyl moiety has been connected through a phosphodiester bond to the metal chelator diethylenetriaminepentaacetic acid (DTPA) for magnetic resonance imaging (MRI) applications[7] and to short peptides.[8] These compounds display dissociation constants ($K_d$) from human serum albumin in the 100 μM range[9] and are susceptible to hydrolysis in vivo.

Thus, there is a considerable scientific and biotechnological interest in the identification of small portable binders that display a stable noncovalent interaction with serum albumin. Herein, we report the discovery and characterization of a class of 4-(*p*-iodophenyl)butyric acid derivatives from a DNA-encoded chemical library,[10] which display a stable noncovalent binding interaction with both mouse serum albumin (MSA) and human serum albumin (HSA). One of these portable albumin-binding moieties was used to improve the performance of the contrast agents fluorescein and Gd-DTPA.

HSA-binding molecules were selected from a DNA-encoded chemical library consisting of 619 oligonucleotide-compound conjugates carrying a six-base-pair code for identification.[11–13] After selection, the DNA sequences of the enriched compounds were amplified by PCR and decoded on oligonucleotide microarrays displaying the complementary sequences (Figure 1a), normalizing the signal intensities after selection against the intensities of compounds selected on empty resin (Figure 1b). Some of the identified binding molecules were excluded from further evaluation based on being promiscuous binders or because of the high standard deviations of the signal intensities on the microarrays (**64**, **313**, **453**, **454**, **619**). Several of the selected molecules (**428**, **533**, **535**, **539**) displayed striking structural similarities. The basic structure featured a 4-phenylbutanoic acid moiety, with different hydrophobic substituents on the phenyl ring.

To obtain further insights into structure–activity relationships, DNA-modified analogues containing propanoyl or pentanoyl skeletons, and/or carrying various substituents on the phenyl ring (Figure 1c; **536**, **622–632**), were characterized in a radioactivity-based chromatographic albumin-binding assay,[12] which allowed a first classification of the potential binders (Retention: **428** > **539** > **624** > **535** > **533** > **536** > **326** > others; see the Supporting Information). The absence of retention of compounds with propanoyl (**625**) and penta-

[*] S. Trüssel, F. Buller, Dr. F. Bootz, Dr. Y. Zhang, L. Mannocci, Dr. J. Scheuermann, Prof. Dr. D. Neri
Institut für Pharmazeutische Wissenschaften
Departement für Chemie und Angewandte Biowissenschaften
ETH Zürich
Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland)
Fax: (+41) 44-633-1358
E-mail: neri@pharma.ethz.ch
Dr. C. E. Dumelin, Dr. E. Trachsel, Dr. S. Melkko
Philochem AG, c/o ETH Zürich
Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland)
Dr. S. C. Beck, Dr. M. Drumea-Mirancea, Dr. M. W. Seeliger
Departement für Augenheilkunde
Forschungsinstitut für Augenheilkunde
AG Neurodegeneration des Auges
Universitätsklinikum Tübingen
Schleichstrasse 4/3, 72076 Tübingen (Germany)
Dr. C. Baltes, Dr. T. Müggler, F. Kranz, Prof. Dr. M. Rudin
Animal Imaging Center, Institut für Biomedizinische Technik
Departement für Elektrotechnik, Universität und ETH Zürich
Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland)

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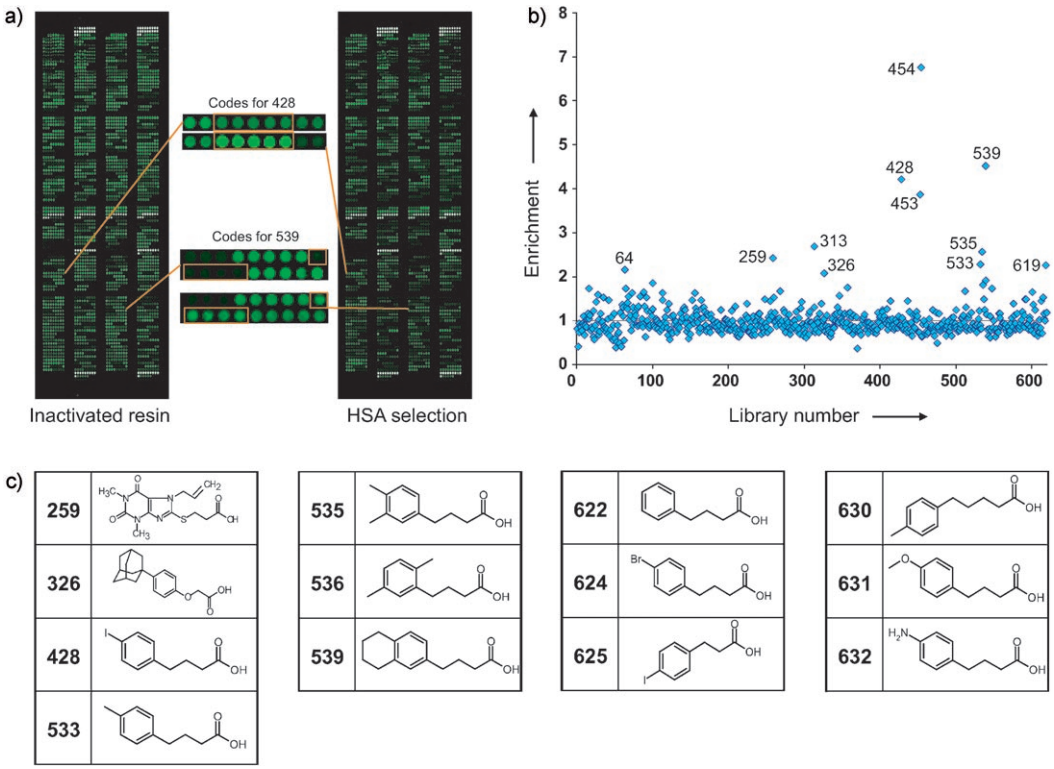


***Figure 1.*** Selection of albumin binders. a) Microarray readout of the selections performed against inactivated resin (left panel) and resin displaying HSA (right panel). The spots corresponding to the enriched compounds **428** and **539** are enlarged (center). b) Enrichment of compounds in selections for HSA binding (compound numbers are indicated). c) Structures of the molecules identified as potential binders.

noyl skeletons (**630**) revealed the essential requirement of a butanoyl moiety for albumin binding. Furthermore, hydrophobic groups in the *para* position of the phenyl ring were found to be important determinants of binding activity, as molecules lacking this feature (**536**, **622**) or carrying hydrophilic *para* substitutents (**631**, **632**) displayed weak or no retention.

Although the DNA-tagged compound **428** showed the highest retention in the chromatographic albumin-binding assay, it exhibited only weak binding in isothermal titration calorimetry (ITC) studies performed in solution. However, stable albumin binding could be recovered upon introduction of a carboxylate group (reminiscent of the terminal 5′ phosphate group connecting the compound to the oligonucleotide) by conjugation of D-lysine to **428** through the ε-amino group (Figure 2a). Modification of the α-amino group of D-lysine with acetic (**428**-D-Lys-Ac, $K_d = 3.2\ \mu M$) or butanoic acid ($K_d = 5.5\ \mu M$) was possible without any substantial change in affinity. **428**-D-Lys-Ac further bound to MSA with a similar affinity ($K_d = 3.6\ \mu M$). Figure 2b shows ITC profiles of several HSA binders conjugated to acetylated D-lysine. All compounds had affinities in the micromolar range, with a ranking of $K_d$ values well in line with the corresponding retention in the chromatographic albumin-capture assay. Further insight into the structure–activity relationship of the carboxylate was obtained by ITC measurements of **428** derivatives with L-lysine and L-ornithine (see the Supporting Information).

Fluorescein and several derivatives (**428**-D-Lys-FAM, **622**-D-Lys-FAM, and phenethylamine-FAM; FAM = 5-carboxyfluorescein) were studied to investigate the applicability of **428**-D-Lys as a portable albumin-binding moiety and with the aim to develop an innovative reagent for angiographic procedures. Fluorescein is widely used in ophthalmology as a contrast agent in fluorescein angiography of the eye fundus in patients with retinal disorders.[14] However, the short circulatory half-life of fluorescein requires injection of high doses (200–500 mg per patient) with nonnegligible side effects[15] and impedes the comparative study of both eyes.

The $K_d$ value of **428**-D-Lys-FAM to albumin measured with ITC, by titrating HSA with **428**-D-Lys-FAM, was 330 nM at 37 °C (Figure 3a), whereas fluorescein displayed no binding to HSA (see the Supporting Information). The affinities of fluorescein and its derivatives at 25 °C were determined by fluorescence polarization (FP) at increasing concentrations of HSA. The $K_d$ values were 76 μM for fluorescein, 108 nM for **428**-D-Lys-FAM, 6.6 μM for **622**-D-Lys-FAM, and 17 μM for phenethylamine-FAM (Figure 3b). The binding affinities of **428**-D-Lys-FAM to HSA and MSA measured by FP were comparable: $K_{d,MSA} = 118$ nM. Additional characterization of the interaction of the FAM derivatives with albumin by band-shift assay confirmed their ability to form a kinetically stable complex with HSA in solution and in serum (Figure 3c). To elucidate the site of interaction of **428**-D-Lys-FAM on HSA its binding was competed with several known ligands (site I, site II, fatty acid, and metal ion), which suggested binding of **428**-D-Lys-FAM at site II (see the Supporting Information).

The pharmacokinetic properties of fluorescein and its derivatives were studied in mice after intravenous (i.v.) injection, and exhibited strikingly different plasma concentration time courses. Fluorescein was no longer detectable 30 min after injection, whereas the derivatives displayed a biphasic pharmacokinetic profile which correlated well with their affinities towards HSA. In particular, the half-life of **428**-D-Lys-FAM was substantially increased and the molecule was still detectable in the bloodstream 24 h after injection

  

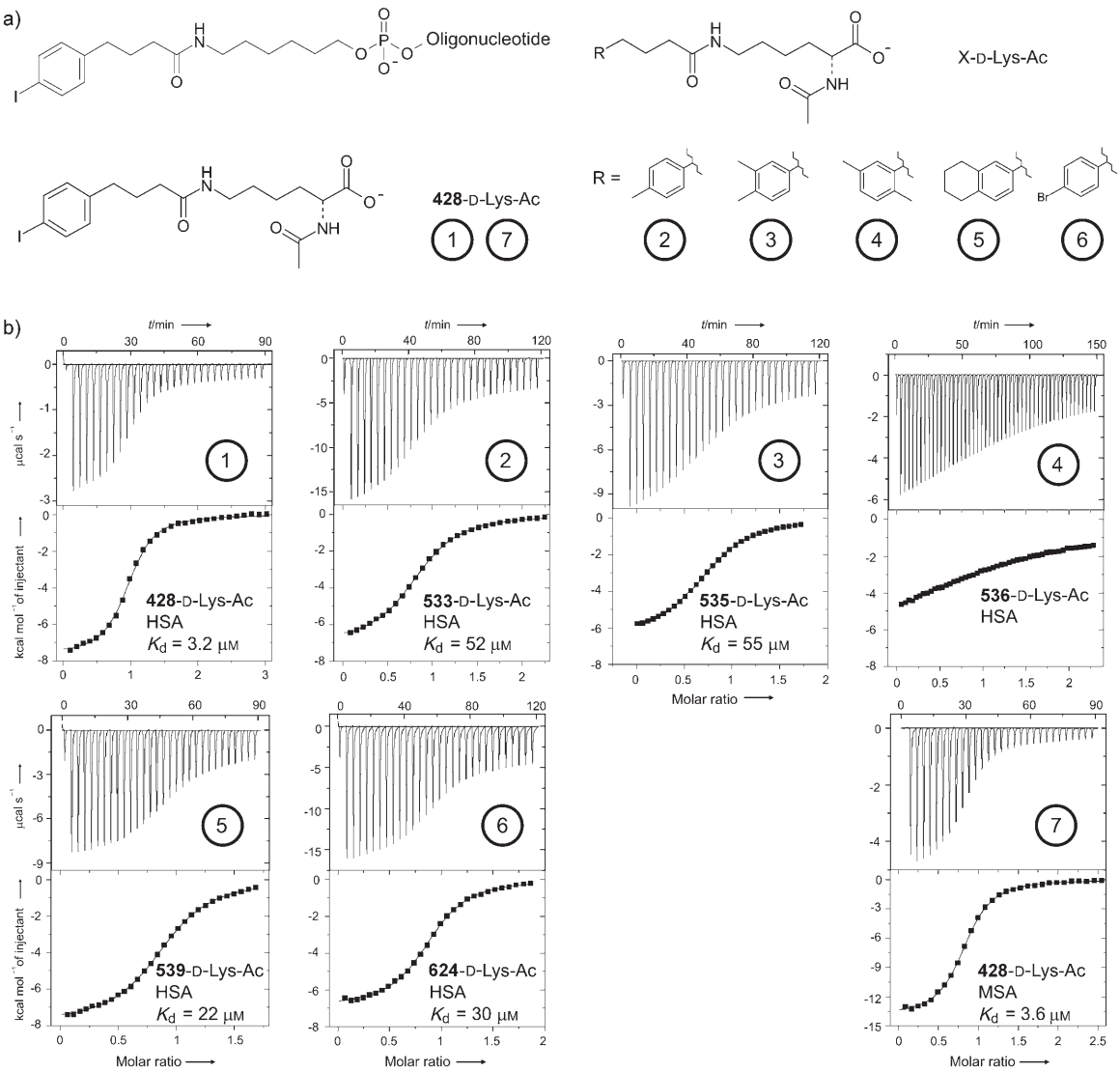


***Figure 2.*** Structure–activity relationship of albumin binders. a) Structures of **428** conjugated to an amino-tagged oligonucleotide and to acetylated D-Lys (**1**, **7**) and a general structure of the molecules measured by ITC (**2**–**6**; X = **533**, **535**, **536**, **539**, and **624**, respectively), which highlight the similar positioning of the negative charge. b) ITC characterization of identified molecules binding to HSA (**1**–**6**) and MSA (**7**).

(Figure 4a; fluorescein: $t_{1/2} = 4.6$ min; **428**-D-Lys-FAM: $t_{1/2,\alpha} = 27$ min, $\alpha$-phase = 90%, $t_{1/2,\beta} = 495$ min; **622**-D-Lys-FAM: $t_{1/2,\alpha} = 5.3$ min, $\alpha$-phase = 98%, $t_{1/2,\beta} = 100$ min; phenethylamine-FAM: $t_{1/2,\alpha} = 5$ min, $\alpha$-phase = 99.5%, $t_{1/2,\beta} = 53$ min).

To gain functional information on the performance of **428**-D-Lys-FAM as a contrast agent, a fluorescein angiographic analysis of the retina was performed in mice. Identical molar amounts of fluorescein and **428**-D-Lys-FAM were applied by i.v. injection and fluorescent images of the eye fundus were recorded at different time points, which confirmed the results of the pharmacokinetic studies. For fluorescein, the staining of the blood vessels was weak after one minute, thus indicating that a substantial reduction of plasma concentration had already taken place. One hour after injection, no fluorescein was detectable in the retinal blood vessels. By contrast, the staining of the blood vessels with **428**-D-Lys-FAM was longer lasting and allowed the visualization of smaller blood vessels (Figure 4b).

To verify the versatility of the albumin-binding molecule, a second contrast agent (Gd-DTPA (Magnevist)) was conjugated to **428**-D-Lys (**428**-D-Lys-βAla-DTPA-Gd). DTPA is commonly used to chelate $Gd^{III}$ and represents the most widely used contrast agent in MRI. The dissociation constant of **428**-D-Lys-β-Ala-DTPA-Gd to HSA was determined by ITC at 37 °C ($K_d = 3.3$ μM, Figure 5a), while Gd-DTPA displayed only little binding to HSA (see the Supporting Information). Pharmacokinetic profiles were studied in mice by injecting DTPA and **428**-D-Lys-βAla-DTPA complexed with $^{177}Lu$, thus allowing quantification by γ-counting. Similar to the situation encountered with the fluorescein derivatives, the plasma concentration of DTPA-$^{177}Lu$ decreased rapidly and was no longer detectable 60 min after injection, whereas **428**-D-Lys-βAla-DTPA-$^{177}Lu$ displayed a substantially slower biphasic pharmacokinetic profile (Figure 5b; DTPA-$^{177}Lu$: $t_{1/2,\alpha} = 8.6$ min; **428**-D-Lys-βAla-DTPA-$^{177}Lu$: $t_{1/2,\alpha} = 22$ min, $\alpha$-phase = 90%, $t_{1/2,\beta} = 408$ min).

  

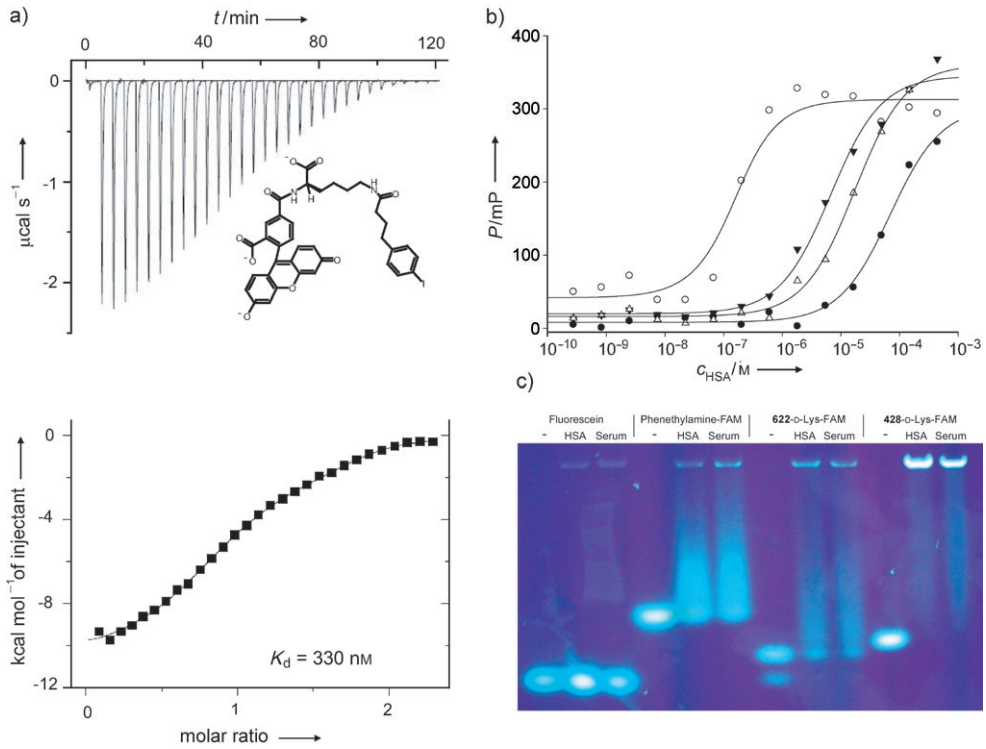


***Figure 3.*** In vitro characterization of fluorescein derivatives. a) ITC profile of HSA titrated with **428**-D-Lys-FAM to determine the $K_d$ value at 37 °C. The structure of the compound is indicated. b) FP of fluorescein (●, $K_d$ = 70 μM), phenethylamine-FAM (△, $K_d$ = 17 μM), **622**-D-Lys-FAM (▼, $K_d$ = 6.6 μM), and **428**-D-Lys-FAM (○, $K_d$ = 108 nM) titrated with increasing amounts of HSA at 25 °C. c) Band-shift assay of fluorescein, phenethylamine-FAM, **622**-D-Lys-FAM, and **428**-D-Lys-FAM in the absence (−) and presence of HSA (purified protein or in human serum).

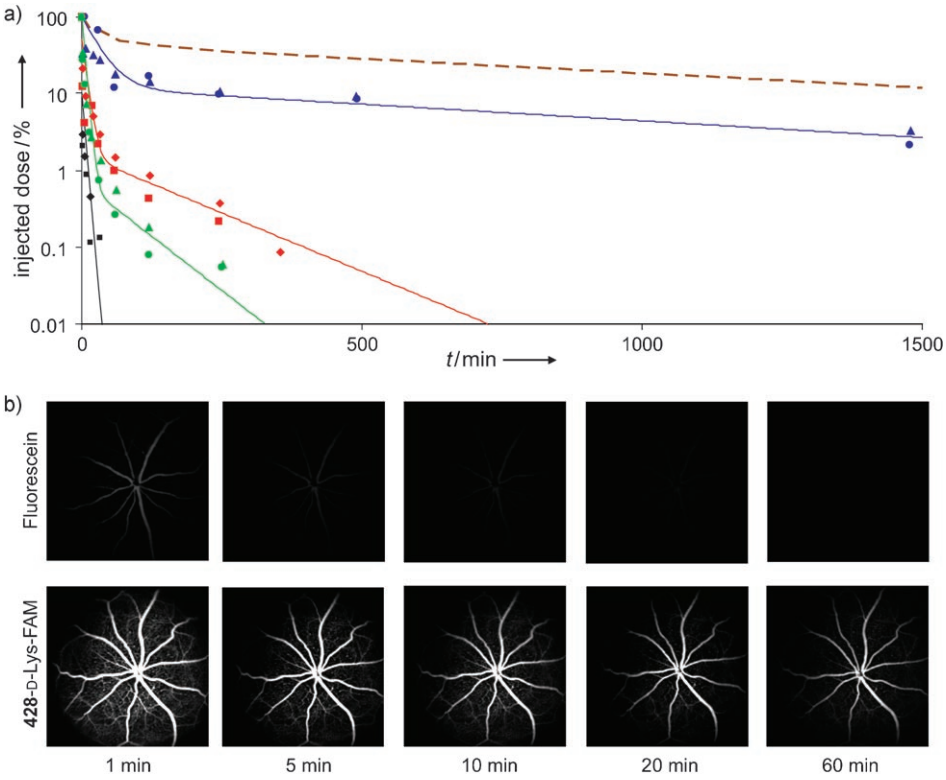


***Figure 4.*** In vivo characterization of fluorescein derivatives. a) Pharmacokinetic studies of fluorescein (black), **428**-D-Lys-FAM (blue), **622**-D-Lys-FAM (red), and phenethylamine-FAM (green) after i.v. injection in two mice each. The plasma concentration time course of $^{177}$Lu-labeled MSA is given for comparison (-----). b) Fluorescein angiography in mice. Images were recorded over 1 h after i.v. injection of 50 nmol of fluorescein (top row) and **428**-D-Lys-FAM (bottom row).

  

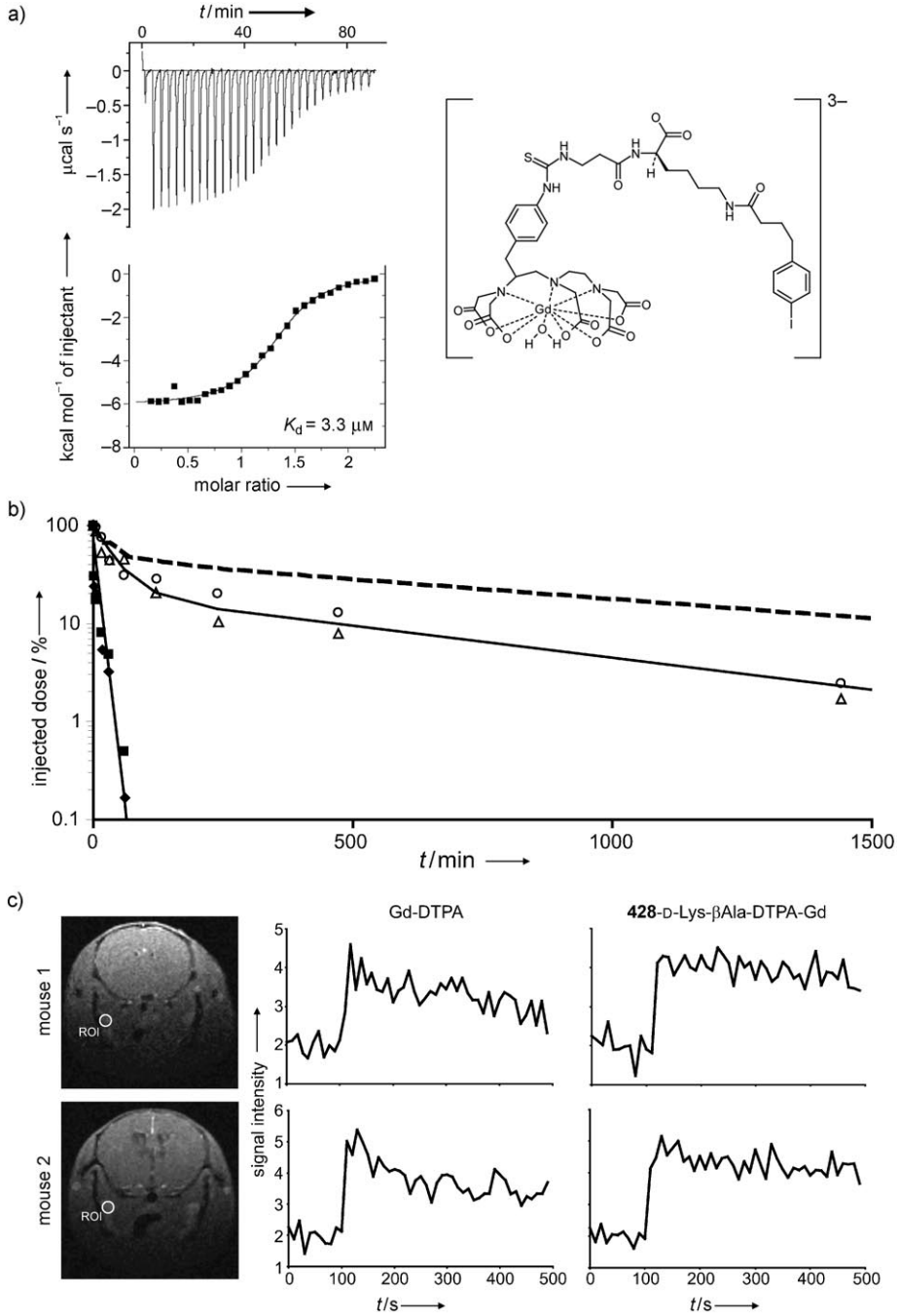


***Figure 5.*** Characterization of DTPA derivatives. a) $K_d$ value of **428**-D-Lys-DTPA-Gd to HSA determined by ITC at 37 °C. The structure of the compound is indicated. b) Pharmacokinetic studies of DTPA-$^{177}$Lu (filled symbols) and **428**-D-Lys-βAla-DTPA-$^{177}$Lu (empty symbols) after i.v. injection in two mice each. The plasma concentration time course of $^{177}$Lu-labeled MSA (-----) is given for comparison. c) Transverse MR images of the mouse head indicating the region of interest (ROI) used to select the blood vessel. Time course of the MR signal intensity in the ROI after injection of Gd-DTPA (left panels) and **428**-D-Lys-β-Ala-DTPA-Gd (right panels).

The rapid extravasation of DTPA-Gd in comparison to **428**-D-Lys-βAla-DTPA-Gd was also observed by MRI procedures following i.v. injection of the contrast agents. MRI analysis of major blood vessels of the head in mice revealed a slower decrease of signal intensities in those injected with **428**-D-Lys-βAla-DTPA-Gd (Figure 5c).

In summary, we have discovered a novel class of albumin-binding derivatives using DNA-encoded chemical libraries capable of forming kinetically stable complexes with both HSA and MSA. The use of **428**-D-Lys as a portable albumin binder was shown to improve the in vivo circulatory half-life of agents of pharmaceutical interest by more than 100-fold. **428**-D-Lys derivatives of fluorescein and of metal ion–DTPA complexes exhibited promising in vivo properties and are likely to represent superior blood-pool contrast agents for clinical applications by increasing the measurement time,

  

reducing the extravasation, and thereby increasing the contrast. For MRI contrast agents the association of $Gd^{III}$ to albumin leads to an enhanced relaxivity as a result of slower tumbling. We anticipate a broad range of biomedical applications for the portable albumin-binding moieties described, particularly for the generation of prodrugs with long circulatory half-lives, for the amelioration of pharmacokinetic properties of therapeutic proteins and peptides, and for blood-pool contrast agents.

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