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COMMUNICATION

Real-Time Interrogation of Aspirin Reactivity, Biochemistry, and Biodistribution by Hyperpolarized Magnetic Resonance Spectroscopy

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Abstract: Hyperpolarized magnetic resonance spectroscopy (HP-MR) enables quantitative, non-radioactive, real-time measurement of imaging probe biodistribution and metabolism *in vivo*. Here, we investigate and report on the development and characterization of hyperpolarized acetylsalicylic acid (aspirin) and its use as a nuclear magnetic resonance (NMR) probe. Aspirin derivatives were synthesized with single- and double-^{13}C labels and hyperpolarized by dynamic nuclear polarization with 4.7% and 3% polarization, respectively. The longitudinal relaxation constants (T_1) for the labeled acetyl and carboxyl carbonyls were approximately 30 seconds, supporting *in vivo* imaging and spectroscopy applications. *In vitro* hydrolysis, transacetylation, and albumin binding of hyperpolarized aspirin were readily monitored in real time by ^{13}C-NMR spectroscopy. Hyperpolarized, double-labeled aspirin was well tolerated in mice and could be observed by both ^{13}C-MR imaging and ^{13}C-NMR spectroscopy *in vivo*.

Recent epidemiologic evidence strongly suggests that long-term low-dose acetylsalicylic acid (aspirin) use significantly decreases the incidence of many cancers.[1] While this effect may be mediated by cyclooxygenase-2 (COX-2) inhibition in the tumor and surrounding stroma,[2] aspirin's highly promiscuous transacetylation activity[3] suggests alternative mechanisms of action.

Aspirin is initially metabolized *in vivo* by hydrolysis of the O-acetyl group (position 8, Figure 1) to form acetate and salicylic acid. Following hydrolysis, salicylic acid is conjugated to glycine or glucuronic acid at the exocyclic carboxyl group (position 7). Aspirin metabolism is heterogeneous and can vary as much as 20-fold among individuals[4], potentially modulating its chemopreventive effect within patient populations[5]. Long-term aspirin use can also result in gastrointestinal bleeding and intracranial hemorrhages in some patients,[6] further complicating its routine use as a cancer chemopreventive.

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Given this metabolic and toxicologic variability, it is desirable to develop a method to monitor aspirin metabolism and biodistribution in real time in patients. While radiotracer-based methods have been used to interrogate aspirin biodistribution and pharmacokinetics,[7] these approaches cannot distinguish between the intact compound and its metabolic byproducts and are poorly adaptable to routine patient imaging.

The advent of hyperpolarized nuclear magnetic resonance (NMR) spectroscopy enables the real-time interrogation of imaging probe metabolism in living systems.[8] In ^{13}C hyperpolarized NMR experiments, a ^{13}C-labeled compound (e.g., [1-^{13}C]-pyruvate) is polarized by one of a variety of methods (e.g., dynamic nuclear polarization [DNP],[9] parahydrogen and synthesis allow dramatically enhanced nuclear alignment [PASADENA][10], or brute force[11]), rapidly injected into the animal or phantom, and its metabolism observed by ^{13}C spectroscopy or spectroscopic imaging.[12]

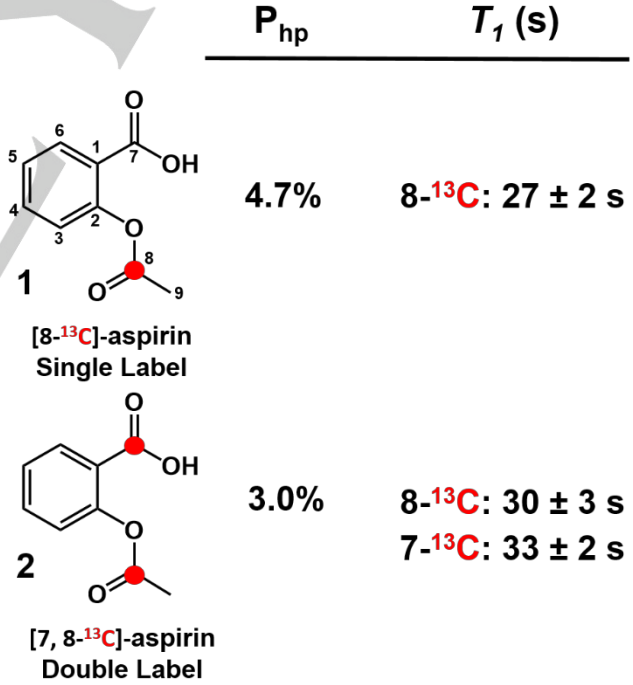

Figure 1: ^{13}C-labeled acetylsalicylic acid (aspirin) derivatives synthesized in this study. ^{13}C-labeled carbons are represented by a red dot. The carbon numbering scheme used throughout this manuscript is shown. Percent polarization values (P_{hp}) and median longitudinal relaxation constants (T_1) for single-labeled (n=2) and double-labeled (n=2) aspirin at 7T are provided ± standard error of the mean.

Here, we report the successful hyperpolarization of ^{13}C-labeled aspirin; real-time monitoring of its *in vitro* hydrolysis,

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transacetylation, and albumin-binding reactions; and visualization of its early biodistribution in mice. This work represents one of the first demonstrations of ^{13}C-NMR spectroscopy as a tool to track the biodistribution of a clinical drug *in vivo* as well as the first use of aspirin as a hyperpolarized MR probe.

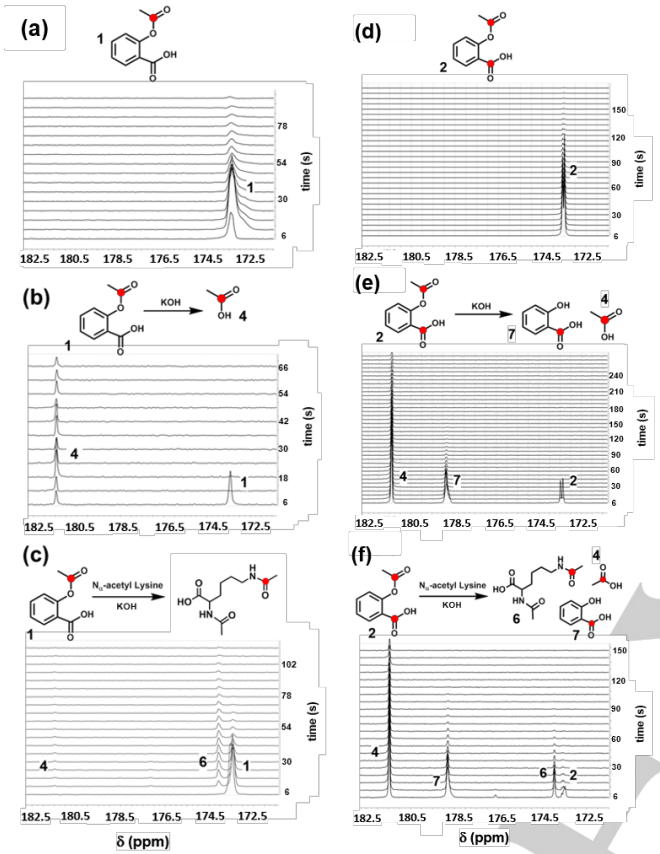

Figure 2. ***In vitro*** **reactions with hyperpolarized (1) and (2). a)** Hyperpolarized **(1)** in phosphate-buffered saline (100% D_2O, 7T). **b)** Hyperpolarized **(1)** in 1 M KOH hydrolyzes rapidly to form hyperpolarized acetate **(4),** seen at 181.5 ppm. **c)** Hyperpolarized **(1)** in 1 M KOH and 250 mM *N*α-acetyl lysine undergoes rapid transacetylation to form *N*α, ^{13}C-*N*ε-diacetyl lysine **(6),** seen at 174.1 ppm. **d)** Hyperpolarized **(2)** in phosphate-buffered saline (100% D_2O). Both ^{13}C-labeled carbons were observed as a partially resolved doublet at 173.3-173.6 ppm (7T). **e)** Hydrolysis of hyperpolarized **(2)** in excess KOH resulted in the formation of ^{13}C-acetate **(4)** and ^{13}C-salicylic acid **(7)** (178.7 ppm). **f)** Reaction with hyperpolarized **(2)** and *N*α-acetyl lysine showed the expected *N*α, ^{13}C-*N*ε-diacetyl lysine **(6)** as well as ^{13}C-acetate and ^{13}C-salicylic acid.

We reasoned that labeling the *O*-acetyl (position 8) and carboxyl carbonyl (position 7) carbons of aspirin with ^{13}C would allow us to monitor both aspirin transacetylation and metabolism *in vivo* by changes in the chemical shift of these nuclei. Single-**(1)** and double-labeled **(2)** aspirin (Figure 1) were readily obtained by the reaction of salicylic acid or labeled [7-^{13}C]-salicylic acid with [1,1-$^{13}C_2$]-acetic anhydride or [1-^{13}C]-acetyl chloride followed by silica gel chromatography and recrystallization from ethanol[13] (Figures S1-S6). Both aspirin derivatives were polarized using DNP (HyperSense, Oxford Instruments). In a typical hyperpolarization experiment, ^{13}C-labeled aspirin was dissolved in a 63% (v/v) dimethyl sulfoxide-d_6 and water solution to a final concentration of 1.5 M. Oxo63 free radical (Oxford Instruments) was added directly to the mixture to a final concentration of 15.6 mM. Approximately 168 μmoles labeled aspirin were hyperpolarized in each experiment with gadolinium III complex (Prohance, 1.8 mM, Bracco Diagnostics) added to each sample to enhance solid-state polarization. Samples were polarized for 1 to 1.5 hours and then dissolved in 4 mL of either phosphate-buffered saline (PBS) in deuterium oxide (pH = 7.2) or PBS in 10% deuterium oxide to a final concentration of ~42 mM hyperpolarized aspirin. Following dissolution, the hyperpolarized material was rapidly injected in either a 7T horizontal-bore MR scanner or a vertical-bore 7T NMR spectrometer (See Figure S7 for a schematic of the experimental setup)

In the case of hyperpolarized **(1),** a single major peak at 173.3 ppm was observed by ^{13}C-NMR spectroscopy (Figure 2a). The chemical shift of the major peak was consistent with the *O*-^{13}C acetyl carbon of intact aspirin (Figure S2). There was no indication of hydrolysis of the hyperpolarized compound after dissolution or during spectroscopy. The percent polarization (P_{hp}) was approximately 4.7% at the time of detection by comparison to the signal from the same, thermally polarized sample. This represents a >3000-fold signal enhancement relative to the unpolarized material at 7T field strength[14]. Double-labeled aspirin **(2)** showed enhanced polarization buildup in the solid state relative to the single-labeled compound, likely resulting from the addition of the second ^{13}C nucleus. ^{13}C-NMR spectroscopy of hyperpolarized **(2)** showed two partially resolved resonances at 173.3 and 173.6 ppm (Figure 2d) corresponding to the *O*-^{13}C acetyl carbonyl (position 8) and carboxylic acid carbonyl carbons (position 7), respectively (Figure S4). The combined polarization for both carbonyls in **(2)** was approximately 3%[14].

The T_1 of the hyperpolarized *O*-acetyl ^{13}C-carbonyl in hyperpolarized **(1)** was measured in 100% D_2O and at 37 ˚C by NMR using a 12° flip angle every 6 seconds and found to be 27 ± 2 seconds in longitudinal relaxation experiments**,** sufficient for approximately 1 minute of imaging *in vivo*. These experiments showed nearly identical values for the *O*-acetyl ^{13}C-carbonyl carbon in hyperpolarized **(2)** (30 ± 3 seconds) and a T_1 of 33 ± 2 seconds for the ^{13}C-labeled carboxylic acid carbon (position 7, Figure 1). Previous work has shown that incorporation of deuterium nuclei within 1-2 bonds of ^{13}C nuclei eliminates relaxation pathways and enhances the T_1 of the ^{13}C label.[15] With this in mind, derivative **(3)**, which is deuterated at the *O*-acetyl methyl carbon, was synthesized and hyperpolarized by DNP. Unfortunately, no significant effect on the T_1 of the *O*-acetyl carbonyl carbon was observed which may indicate that the methyl protons of aspirin do not play a significant role in the spin-lattice relaxation of this nucleus (Figure S8).

We next sought to determine whether hydrolysis and transacetylation reactions with hyperpolarized **(1)** could be observed by hyperpolarized ^{13}C-NMR. Initial NMR and mass spectrometry studies of non-hyperpolarized, ^{13}C-labeled aspirin showed rapid transacetylation of glycine and Nα-acetyl lysine (Figures S9 and S10)**,** as evidenced by the appearance of peaks at 173.9 ppm and 173.8 ppm corresponding to acetylation of the α-amino group of glycine and the Nε-amino group of Nα-acetyl lysine, respectively. The chemical shifts of the acetylated products are well-resolved from the intact aspirin *O*-acetyl carbonyl resonance at 173.3 ppm and are consistent with those

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observed in previous transacetylation studies with non-hyperpolarized ^{13}C-labeled aspirin.[16] Reaction of hyperpolarized **(1)** with excess KOH (pH = 10-11) showed rapid hydrolysis to acetate (Figure 2b). In contrast, no hydrolysis was observed at biological pH (pH = 7.2). Addition of Nα-acetyl lysine (Figure 2c) in the presence of hyperpolarized **(1)** and excess KOH showed rapid and complete conversion to the expected *N, N*-diacetyl product within approximately 20 seconds. A parallel set of experiments with hyperpolarized **(2)** showed rapid conversion to acetate and salicylic acid (181.3 and 178.7 ppm, respectively) in the presence of KOH (Figure 2e) and conversion to acetate, salicylic acid, and Nα, ^{13}C-Nε-diacetyl lysine in the presence of KOH and Nα-acetyl lysine (Figure 2f). The resonances for acetate and salicylic acid were well resolved from the parent resonance ($\Delta\delta$ = 8.0 and 5.4 ppm respectively), suggesting that these products can be readily detected *in vivo* by MR spectroscopy. The T_1 values for aspirin derivatives and reaction products (Table S1) were similar to those of the carbons at positions 7 and 8 of aspirin, while the T_1 of the acetate carbonyl carbon was significantly higher, as expected on the basis of previous studies.[17] These experiments demonstrated that transacetylation and hydrolysis reactions of both hyperpolarized aspirin derivatives could be monitored in real time by ^{13}C-NMR on the time scale of the hyperpolarized signal.

In preparation for animal studies, we investigated the interaction of hyperpolarized aspirin with serum albumin using hyperpolarized NMR. Human albumin contains multiple aspirin-binding sites[18] and is covalently acetylated by aspirin at lysine residues in a pH-dependent manner.[19] Incubation of hyperpolarized **(1)** with a 5% solution of bovine serum albumin (BSA) in PBS resulted in the appearance of a broad resonance centered at 172.9 ppm that was partially resolved from a sharp minor resonance at 173.1 ppm (Figure 3a). The linewidth of the broad resonance was 15 Hz, and the T_1 was approximately 15 seconds. In contrast, the linewidth of the sharp minor resonance was 5 Hz, consistent with hyperpolarized **(1)** in PBS (Figure 3b). Analogous ^{13}C-NMR experiments with non-hyperpolarized **(1)** showed a similar line-broadening effect in the presence of BSA which was significantly reduced by competition with excess ^{12}C-aspirin (Figures 3c, d).

These experiments suggest that the increased linewidth and relaxation rate of **(1)** in the presence of BSA arises from exchange between the free and bound form. This finding is consistent with previous studies showing that the aromatic protons of aspirin undergo increased line-broadening and T_1 reduction upon albumin binding,[20] likely owing to a decreased tumbling rate in the bound form. Further support for exchange-mediated line broadening is seen in the decreased linewidth of **(1)** in ^{12}C-aspirin competition experiments where binding site occupancy by the unlabeled aspirin inhibits exchange of **(1)** with the protein. Although these observations support exchange-mediated line-broadening, we cannot rule out contributions from dipolar coupling between bound **(1)** and BSA.

Finally, we sought to monitor the *in vivo* biodistribution and metabolism of hyperpolarized **(2)**, which offered the possibility of monitoring metabolism at both the *O*-acetyl and carboxylic acid groups. Normal male nude mice were injected with 200 μL of hyperpolarized material (42 mM, 3% polarized) via a tail-vein catheter. An 8-M ^{13}C-urea phantom was used for chemical shift referencing. ^{13}C imaging was performed using an echo-planar imaging (EPI) sequence, and hyperpolarized signal was observed in the inferior vena cava (<5 seconds post-injection) and the heart (>5 seconds post-injection) (Figures 4a and b).

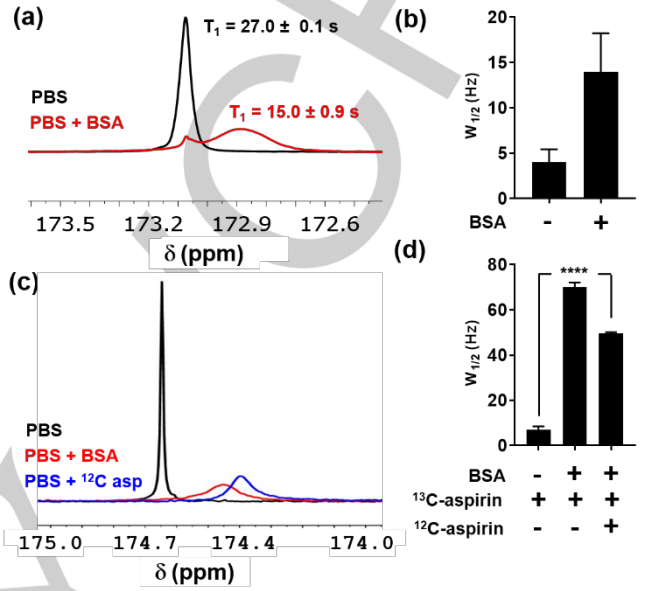

Figure 3. Interaction between hyperpolarized (1) and bovine serum albumin (BSA). a) Stacked ^{13}C-NMR spectra of hyperpolarized **(1)** in phosphate buffered-saline (PBS, 10% D_2O) and 50 mg/mL BSA in PBS (10% D_2O). Median T_1 values for the major resonances in each spectrum are shown ± standard deviation (n = 2). **b)** Average linewidths of the major peaks ($W_{1/2}$ in Hz) in each hyperpolarized spectrum are shown along with the standard deviation (n = 2). **c)** Stacked non-hyperpolarized ^{13}C-NMR spectra of **(1)** in PBS, PBS+BSA, and PBS+BSA with 3-fold excess ^{12}C-aspirin (1000 scans per spectrum). **d)** Median linewidths of the major aspirin resonance in each spectrum along with the standard deviation (n=4). Two-way analysis of variance (ANOVA, multiple comparisons) was used to assess statistical significance (****, $P < 0.0001$).

Images with an acceptable signal-to-noise ratio could be acquired for up to 10 seconds following injection of the hyperpolarized material. A series of slice-selective ^{13}C spectra were collected 1-10 seconds after injection of hyperpolarized material. A single slice was placed over the majority of the animal, and single free induction decay (FID) signals were acquired every 2 seconds. The *in vivo* spectroscopy showed the expected double peak of **(2)**, but no hydrolysis or transacetylation products were observed (Figures 4c and d). The full time courses of both imaging experiments are shown in Figures S11 and S12 and enlarged versions of 4a and 4b are shown in Figures S13 and S14.

Both ^{13}C-labeled aspirin derivatives synthesized in this work were efficiently polarized by DNP within 90 minutes and retained this polarization after dissolution. The longitudinal relaxation times for both ^{13}C-labeled carbonyls were approximately 30 seconds, which was sufficient for *in vivo* imaging and spectroscopy. *In vitro* base-catalyzed hydrolysis and transacetylation reactions of both hyperpolarized derivatives could be followed in real time by NMR spectroscopy, suggesting that this technique could be used to observe analogous reactions *in vivo*. Binding of hyperpolarized **(1)** to serum albumin was readily observed by changes in

linewidth, relaxation time, and chemical shift, suggesting that hyperpolarized NMR spectroscopy can be used for label-free real-time analysis of aspirin-protein binding interactions. This finding is consistent with previous work showing the utility of hyperpolarized ^{19}F-NMR for the study of ligand-protein binding interactions in the slow- and fast-exchange regimes.[21]

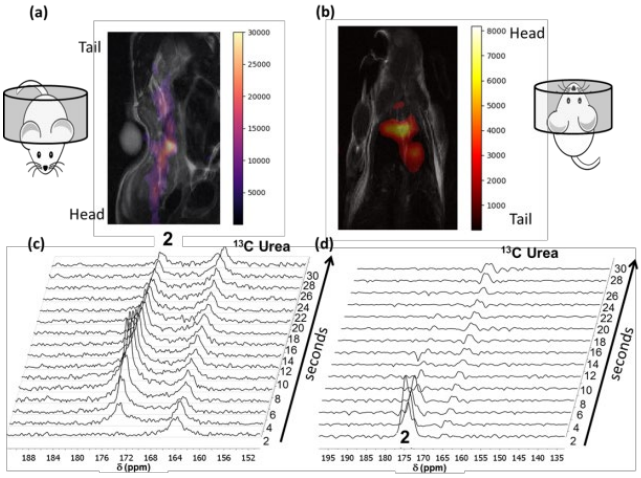

Figure 4. *In vivo* imaging and spectroscopy of hyperpolarized double-labeled aspirin **(2)**. **a)** Overlaid ^{1}H and ^{13}C (false color) coronal image immediately after injection of hyperpolarized **(2)**. The posterior of the mouse was placed inside the volume coil. **b)** Same as a) except the anterior of the mouse occupies the volume coil and imaging was initiated 5 seconds after injection. Single ^{13}C transient arrays following injection of hyperpolarized **(2)** with posterior **c)** or anterior **d)** of the mouse placed in the volume coil. The resonance for **(2)** is readily observed at 174.5 ppm for approximately 30 seconds when spectroscopy is initiated immediately after injection **(c).**

Although we observed the parent hyperpolarized aspirin resonance in mice using *in vivo* MR spectroscopy, there was no evidence of hydrolysis or transacetylation within in any single spectrum or in a summation spectrum of the first 15 individual spectra (Figure S15). This result may be attributable to the low initial polarization of **1** (3%), or the rapid decay of the ^{13}C signal *in vivo* which significantly reduced the imaging time available. We suspect that this signal decay arises from reduction of the *in vivo* T_1 as a result of serum protein binding. Indeed, incubation of hyperpolarized **(1)** with mouse plasma and intact red blood cells showed ~50% reduction in the T_1 of the parent ^{13}C resonance (Figure S16), which is consistent with the albumin-binding experiments (Figure 3a). Additional enhancement of P_{hp} through microwave-gated DNP[22] or the use of clinical polarizers[23] may provide sufficient input signal to compensate for the increased relaxation rate of hyperpolarized aspirin *in vivo*.

To our knowledge, this study is the first demonstration of the use of hyperpolarization to directly visualize *in vitro* albumin binding and early *in vivo* biodistribution of a clinical drug. Previous work with hyperpolarized drugs[24] has not been extended to *in vivo* studies, owing in part to the low T_1 values and dose-limiting toxicities of the hyperpolarized species. Hyperpolarized aspirin maintained an acceptable T_1 in biological mixtures, and mice were able to tolerate multiple injections over a 2-week period (n = 3) with no apparent adverse effects. These results underscore the potential of hyperpolarized NMR to quantitatively and noninvasively assess the metabolic, biochemical, and pharmacologic activity of aspirin *in vitro* and *in vivo*.

Experimental Section

Detailed synthetic protocols and experimental methods along with Figures S1-S16 and Table S1 are available in the Supporting Information document.

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Keywords: aspirin • hyperpolarization • magnetic resonance imaging • magnetic resonance spectroscopy • chemopreventive

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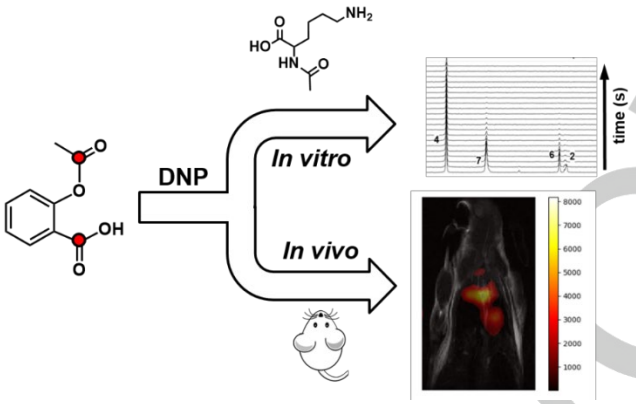
A new trick for an old drug: Zacharias, Ornelas, and co-workers demonstrate the successful hyperpolarization of ^{13}C-labeled aspirin by DNP. NMR spectroscopy was used to monitor *in vitro* hydrolysis, transacetylation, and albumin-binding reactions of hyperpolarized aspirin in real-time and MRI was employed to visualize its early biodistribution in mice.



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