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COMMUNICATIONS

Published on Web 09/03/2005

# A Steroid-Conjugated Contrast Agent for Magnetic Resonance Imaging of Cell Signaling

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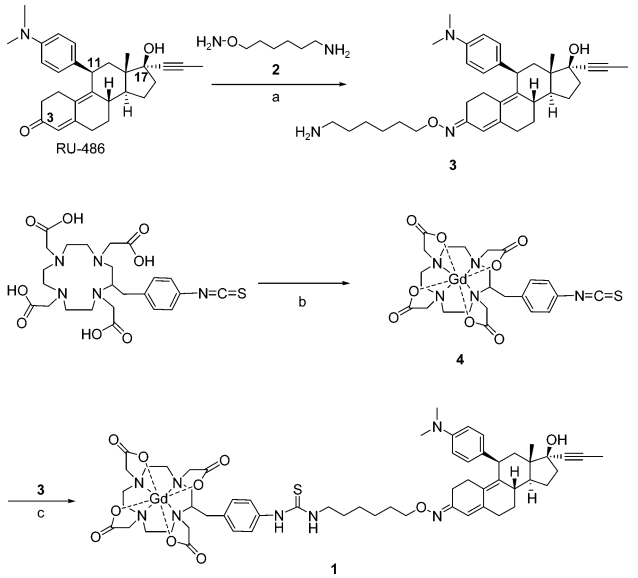
One of the most challenging areas of research in cell biology is tracking cell signaling pathways to understand mechanisms. In an effort to elucidate cell signaling mechanisms, transcriptional systems that can be activated by exogenous molecules have been developed. The GAL4-UAS transcriptional system has a truncated form of the progesterone receptor (PR) hormone-binding domain fused to a gene transactivation domain.[1] The mutated receptor fails to bind progesterone yet retains the ability to bind the synthetic progesterone antagonist RU-486 (mifepristone, Scheme 1). The binding of the mutated progesterone receptor to RU-486 triggers a signal transduction cascade by activating transcription of lac-Z, the target reporter gene. This gene switch system is particularly useful for tracking the cell signaling in vitro and in vivo with an appropriate imaging strategy.[1]

The most popular and general method for imaging cell signaling is light microscopy, which employs the use of organic dyes or fluorescent reporter proteins.[2] However, these techniques are limited by light scattering, frequently produce photobleaching byproducts, and require invasive data collection. Magnetic resonance imaging (MRI) can overcome these problems while visualizing intact opaque organisms in three dimensions and, therefore, provide an alternative to light microscopy and radiopharmaceutical methods.[3−5] The image is based upon the NMR signal from the protons of water, where the signal intensity in a given volume element is a function of the water concentration and relaxation times ($T_1$ and $T_2$).[5] Gadolinium-based contrast agents for MRI enhance tissue contrast by increasing the relaxation rate ($1/T_1$) of water protons and are widely used in clinical diagnostics. When a contrast agent binds to macromolecules, such as enzymes or proteins, the relaxation rate of the protons increases dramatically. Binding to a macromolecule increases concentration and retention of the Gd(III) complex at the receptor binding site and affords an increase in relaxivity as a result of a decrease in rotational correlation time ($\tau_r$).[6]

Conventional MR contrast agents are primarily extracellular and are constitutively detectable.[7] The advent of entirely new classes of agents with increasing sophistication for targeting and bioactivation are expanding the types of experiments performed by MRI.[4] As part of our research in the development of multimodal and multifunctional MR probes, we have prepared several classes of chelates that aid in the detection of biochemical events, including cell permeable, enzyme activated, and intracellular messenger agents.[8]

***Scheme 1.*** Synthesis of a Gd(III) Chelate Conjugated to RU-486 (**1**)[a]




[a] (a) EtOH, 54%; (b) $GdCl_3$, $H_2O$, pH 6−6.5, 83%; (c) DMSO, TEA, 65%.

In this report, we introduce the first steroid hormone−MR contrast agent conjugate that is designed to monitor the activation of a specific signal transduction pathway by MR imaging. We have synthesized a contrast agent covalently attached to RU-486. The Gd(III) chelate conjugated to RU-486 selectively binds to the mutant receptor and activates the endogenous gene expression pathway. In addition, it is expected to increase the rotational correlation time ($\tau_r$) upon binding to the receptor protein resulting in further enhancement of the contrast.

The RU-486−Gd(III) complex (**1**) was synthesized by conjugation of the steroid and macrocycle through an aminooxy-functionalized linker, as shown in Scheme 1. We selected the 3-keto position of RU-486 as the site of modification because it has been reported that the functional groups available for modification, such as 11- and 17-, would significantly affect the biological activity of the steroid.[9] Therefore, a number of strategies were attempted for modification of the ketone group, including reduction and functional group conversion. We began with reduction of the 3-keto group using 9-BBN and coupling reactions through the −OH group using a variety of coupling reagents. In each case, the reaction did not proceed or produced the 3-OH eliminated byproduct. Subsequent Mitsunobu reaction with 3-OH did produce a 3-azido compound in poor yield. All attempts to reduce the azide were unsuccessful because the reduced amine was too unstable to isolate. Direct

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**10.1021/ja051294x CCC: $30.25** 

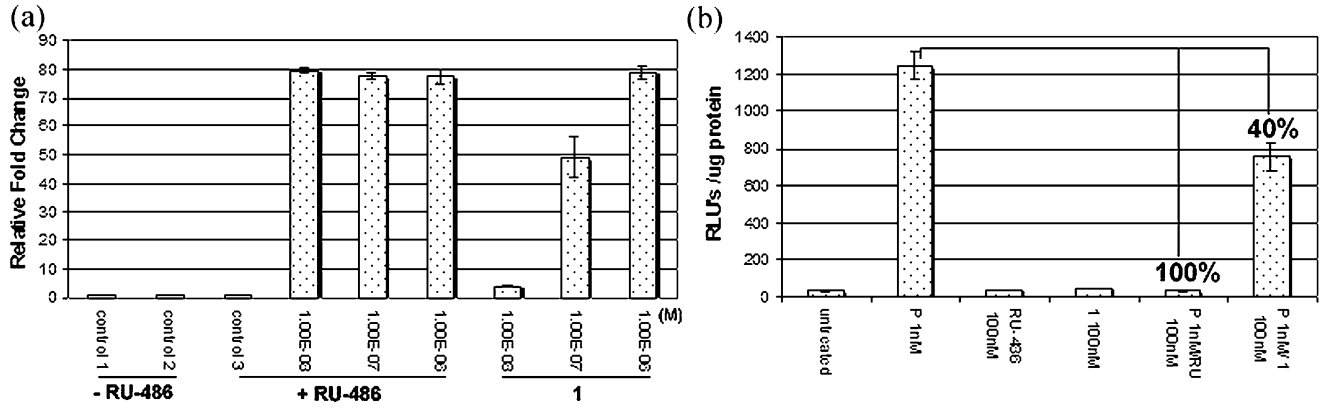


***Figure 1.*** (a) Transcription assay of **1** with GAL4-UAS system. The modified compound **1** showed a successful activation of gene transcription yet 1/100 less $\beta$-galactosidase activity than RU-486; control1-UAS without PR, control2-UAS with PR, control3-UAS without PR treated with $10^{-8}$ M of RU-486. (b) PRE-Luciferase transcription assay of **1**. **1** was able to interact with progesterone receptors in T47D cells; however, it inhibited luciferase transcription less effectively than RU-486.

derivatization of the 3-keto to 3-oxime using hydroxylamine hydrochloride was attempted. However, the 3-oxime was not nucleophilic enough to react with the isothiocyanate group of **4** even when Cu(I)Cl was used as a catalyst.[10] The direct attachment of the aminooxy group containing a bifunctional linker (**3**)[11] followed by the conjugation through the amine yielded the desired product **1**. **1** consists of two isomers of syn and anti with the ratio of 2:3. We attempted separation of the mixture by applying several different strategies. However, in contrast to oxime compounds, it was impossible to separate syn/anti isomers of the aminoxyalkyl derivatives.[12] Therefore, the compound was tested as an isomeric mixture of the syn- and anti-forms isolated from the synthesis.

The relaxivity of **1** ($R_1$) was obtained by taking the slope of a plot of $T_1^{-1}$ versus concentration. Measurements were taken at 60 MHz in deionized water at 37 °C. The relaxivity value was 8.5 $mM^{-1}$ $s^{-1}$, which is more than 2 times the observed value of typical gadolinium chelates, Gd(III)DOTA and Gd(III)DTPA.[7] This higher relaxivity may be due, in part, to torsional strain of the macrocycle resulting in a higher number of coordinated water molecules. Alternatively, the hydrophobic nature of the RU-486 makes the complex amphiphilic enough to aggregate and may result in an increase in the rotational correlation time and subsequently an increase in relaxivity.

Gene activation tests were performed, and the results with GAL4-UAS demonstrated that compound **1** successfully activated transcription at 1/100 native RU-486 (Figure 1a). We then tested the molecule using the progesterone response element (PRE)-luciferase transcriptional activation assay (Figure 1b). Compound **1** inhibited progesterone-mediated transcription with only 40% inhibition of 1 nM progesterone-induced activity and 7% inhibition of 10 nM progesterone, while the same amount of RU-486 resulted in 100% inhibition of 1 nM progesterone and 99% inhibition of 10 nM progesterone. These data suggest that **1** can cross the cell membrane and interact with the receptor in such a way as to antagonize progesterone. Furthermore, these experiments were normalized to protein levels indicating that toxicity associated with the compound is minimal and does not negate transcriptional regulation. The lower activity may be due to the presence of the macrocycle, membrane permeability, or the chelator may hinder receptor binding and affinity.

To determine the factors affecting lower transcriptional activity, we performed a binding affinity test using the progesterone receptor competitor assay kit (Invitrogen) (Figure 2). Interestingly, we found that **1** was 100-fold lower with respect to binding affinity and

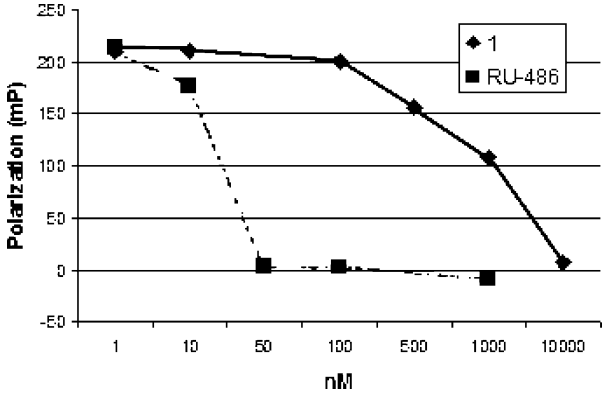


***Figure 2.*** Progesterone receptor binding assay. The calculated $IC_{50}$ values, using a no competitor control as 100% and the no PL ligand as 0%, gave values of 21.91 nM for RU-486 and 1.907 $\mu$M for **1**. These data indicate that compound **1** binds with roughly 100-fold less affinity to the receptor as unconjugated RU-486.

transcriptional activity as compared to that of RU-486 (i.e., binding affinity is proportional to gene transcriptional activation).

Our strategy to modify the 3-position of RU-486 with a contrast agent was based on the assumption that this would not affect the ability to activate GAL4-PR. However, this modification appears to alter the conformation of the steroid and cause partial loss of binding affinity to the receptor protein. One reason for the loss of affinity may be because the 3-keto group is important for hydrogen bonding with the receptor protein.[9a,13] It has been reported that there is a sizable pocket underneath the steroid D-ring in the receptor.[9b] Therefore, modification of the 16- or 17-position of the D-ring with the contrast agent may enable restoration of binding affinity.

To determine the cell permeability and toxicity of **1**, we performed cellular uptake studies by incubating **1** with PR-transfected cells (T47D) and PR-negative cells (MDA-MB-231) (Figure 3). We observed moderate selectivity toward PR positive cells. We also checked for cell viability using a trypan blue assay. There was an average of 1 500 000 cells per flask, and >90% of the cells remained viable after exposure to **1**. These experiments confirm the cell membrane permeability of **1** and indicate that the cellular uptake of **1** was affected by incubation time and concentration of the compound. To determine the effect of **1** on $T_1$ enhancement upon receptor binding, we measured spin−lattice relaxation time ($T_1$) of cells incubated with the contrast agent (Table 1). To get sufficient amounts of the contrast agent into cells, we incubated T47D cells with 50 $\mu$M of **1** and RU-486 for 16 h. **1**

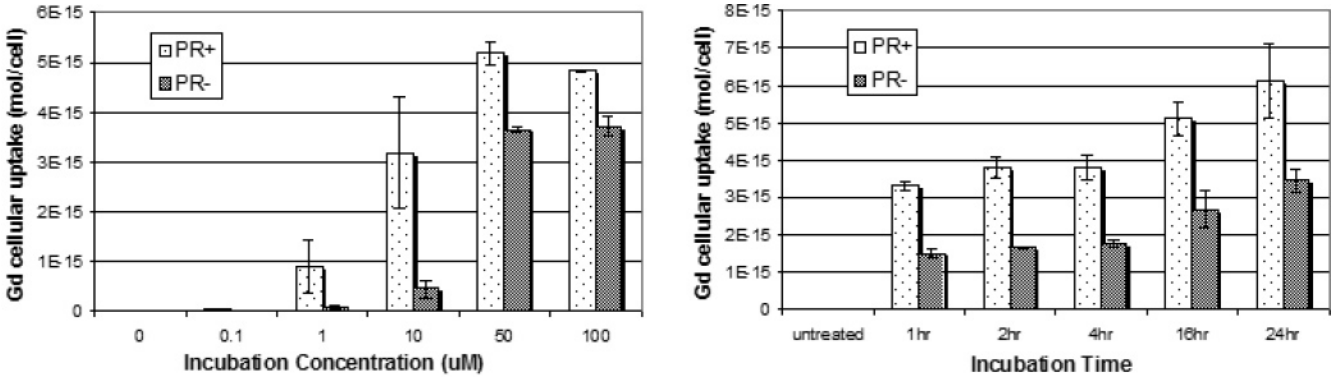


***Figure 3.*** Cellular uptake studies of **1** in PR(+) and PR(−) cells. Compound **1** showed more uptake in PR(+) cells than PR(−) cells.

***Table 1.*** $T_1$ (seconds) of Cells at 9.4T, Where Cells Were Treated with 50 $\mu$M of Each Compound and Incubated for 16 h[a]

| cells/compounds | $T_1$ (s) |
|---|---|
| PR(+) cells treated with RU-486 | $3.18 \pm 0.11$ |
| PR(−) cells treated with RU-486 | $2.90 \pm 0.11$ |
| PR(+) cells treated with **1** | $2.06 \pm 0.16$ |
| PR(−) cells treated with **1** | $2.30 \pm 0.30$ |
| media | $3.03 \pm 0.16$ |

[a] $T_1$-weighted spin−echo MR images are provided in the Supporting Information.

treated cells appear bright and have approximately 40% shorter $T_1$; on the contrary, RU-486 treated cells and media have longer $T_1$. The 40% $T_1$ difference should provide sufficient contrast for in vivo imaging.[8a]

In summary, we have synthesized the first steroid hormone−MR contrast agent conjugate designed to track cell signaling processes upon binding to a transcriptional system. The compound successfully crossed cell membranes and modulated gene transcription. The high relaxivity of **1** showed the contrast enhancement at low concentrations in vitro and did not significantly affect cell viability. By combining a transcriptional system and a noninvasive imaging technology, such as MRI, it should be possible to simultaneously and quantitatively activate transcription of reporter genes and image genetically defined neuronal circuits that express GAL4-PR.[1,14] This should facilitate functional neuroanatomical studies, such as electrophysiology and neuronal tract tracing. We are optimizing the molecular architecture and the incubation conditions to improve the binding affinity and the selective retention.

**Acknowledgment.** We thank Scott Fraser for helpful discussions, Keith MacRenaris for ICP-MS analysis, Steve Bull for image acquisition and $T_1$ analysis, and Ken Korach for providing PRE-luciferase. This work is supported by the National Institutes of Health (5RO1 A147003, DOD 91008600, and PO1HD021921), and by Northwestern University Reproductive Biology Training Grant T32 HD007068.

**Supporting Information Available:** Experimental procedures, spectroscopic data, and $T_1$-weighted MR images. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Two different isomers (syn/anti) of 3-oxime compound were synthesized when RU-486 was refluxed with hydroxylamine hydrochloride in pyridine. The isomers were separated by column chromatography on silica using the eluent of hexane:ethyl acetate = 1:1.

(13) Eliminating isomerism (syn/anti at C3) may enhance the binding affinity. We attempted to separate the isomeric mixture to determine (if any) the effect that a purified isomer may have; however, we were unsuccessful.

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JA051294X