



Monitoring DNA–Ligand Interactions in Living Human Cells Using NMR Spectroscopy

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S Supporting Information

ABSTRACT: Studies on DNA–ligand interactions in the cellular environment are problematic due to the lack of suitable biophysical tools. To address this need, we developed an *in-cell* NMR-based approach for monitoring DNA–ligand interactions inside the nuclei of living human cells. Our method relies on the acquisition of NMR data from cells electroporated with preformed DNA–ligand complexes. The impact of the intracellular environment on the integrity of the complexes is assessed based on *in-cell* NMR signals from unbound and ligand-bound forms of a given DNA target. This technique was tested on complexes of two model DNA fragments and four ligands, namely, a representative DNA minor-groove binder (netropsin) and ligands binding DNA base-pairing defects (naphthalenophanes). In the latter case, we demonstrate that two of the three *in vitro*-validated ligands retain their ability to form stable interactions with their model target DNA *in cellulo*, whereas the third one loses this ability due to off-target interactions with genomic DNA and cellular metabolites. Collectively, our data suggest that direct evaluation of the behavior of drug-like molecules in the intracellular environment provides important insights into the development of DNA-binding ligands with desirable biological activity and minimal side effects resulting from off-target binding.

DNA is an important target for drug design and development.¹ Thus far, the development of DNA-binding ligands has relied on data from *in vitro* biophysical assays, *in vivo* or *in cellulo* biological assays, or *in silico* structure-based DNA-targeting approaches. While biological assays are indispensable for evaluating the biological effects of ligands, their principal disadvantage is the difficulty in connecting the ligand-induced biological effects to the direct action of the ligand on its putative DNA target. In contrast, both *in vitro* and *in silico* approaches are based on the quantitative characterization of a simple two-component

(DNA–ligand) system under non-native conditions. They allow characterization of the DNA–ligand complex in terms of either the structure or quantitative parameters such as the thermodynamic stability of the complex and the kinetics of its formation.^{2–10} Notably, both *in vitro* and *in silico* approaches provide information on DNA–ligand interactions in the absence of the cellular environment. As a consequence, ligand properties determined under these conditions do not automatically reflect the efficacy that the ligands display *in vivo*, where the presence of alternative targets and various environmental factors can influence the formation and stability of DNA–ligand complexes. Undoubtedly, the development of novel tools allowing direct evaluation of drug–target interactions under complex conditions in living cells is of general interest. Here, we elaborate on the recently introduced electroporation concept devised for proteins¹¹ and nucleic acids¹² and extend this approach to characterizing DNA–ligand interactions in the nuclei of living human cells.

The application of *in-cell* NMR spectroscopy for investigating DNA–ligand interactions was tested using two model systems comprising the complexes formed between (i) a 24-nt DNA hairpin (MH-DNA) and netropsin, a polyamide ligand representative of minor-groove DNA binders,^{13–15} and (ii) an 11-bp DNA duplex containing a T·T mismatch (TT-DNA) and a set of three substituted naphthalenophanes 1–3, which are representative of drugs targeting DNA base-pairing defects (Figure 1).^{16,17} These models were chosen because, beyond their potential therapeutic importance,^{15,16} the NMR signals of their ligand-bound and unbound DNA targets are clearly distinct, making them well suited for a proof-of-principle demonstration of the *in-cell* NMR approach. First, we evaluated the efficacy of electroporation-based delivery of the NMR substrate. Notably, two-step delivery of a ligand and its DNA target to cells via two sequential electroporation procedures resulted in high cell mortality and/or DNA leakage from cells independent of the order of component delivery (i.e., DNA

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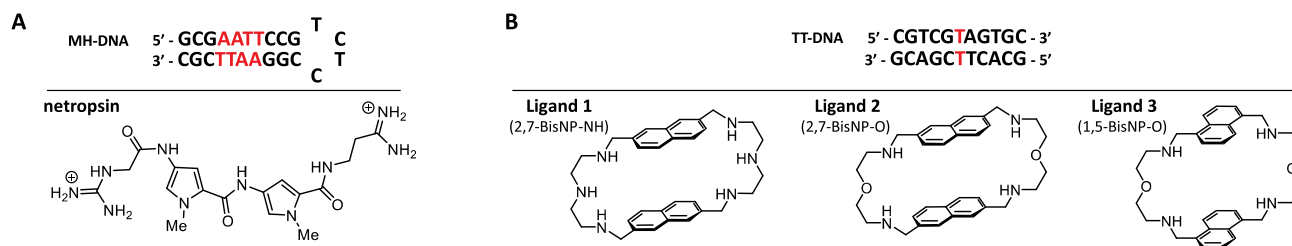


Figure 1. Schematic representations of the DNA targets and DNA-binding ligands used in the present study. (A) Netropsin and its model target, hairpin-based DNA (MH-DNA). (B) Three substituted naphthalenophanes and their model target, T·T mismatch-containing DNA (TT-DNA). Ligand binding sites are indicated in red.

and ligand) (Figure S1A–C). In contrast, the delivery of complexes preformed in the electroporation (EC) buffer proved to be highly effective (Figure S1D): The complexes were introduced into more than 92% of cells at concentrations sufficient for their detection by NMR (Figures 2 and S2).

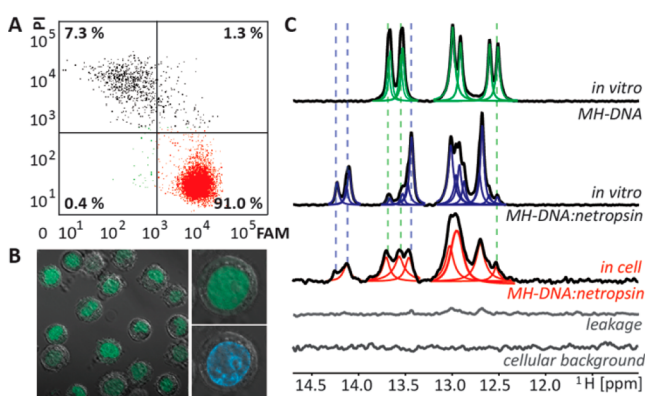


Figure 2. Double-staining (PI/FAM) FCM analysis (A) and confocal microscopy images (B) of cells cotransfected with the (FAM)-MH-DNA:netropsin (1:1) complex. In panel (A), the percentages of viable nontransfected cells, viable MH-DNA containing cells, dead/compromised nontransfected cells, and dead/compromised transfected cells are indicated in the bottom-left, bottom-right, top-left, and top-right quadrants, respectively. In panel (B), the green color marks the localization of (FAM)-MH-DNA, while the blue color marks cell nuclei stained with Hoechst 33342. (C) Deconvoluted imino regions of 1D ¹H NMR spectra of MH-DNA *in vitro* and the 1:1 MH-DNA:netropsin complex *in vitro* and in cells. NMR spectra of extracellular fluid taken from the sample after *in-cell* NMR spectral acquisition and of nontransfected cells (cellular background) are shown in gray. The vertical green and blue dashed lines mark imino signals specific to the unbound and ligand-bound forms of MH-DNA, respectively.

Importantly, among the cells transfected with the complexes (i.e., those contributing to the *in-cell* NMR signals), over 98% remained viable after the time required for the acquisition of NMR spectra (Figure S2).

Moreover, confocal microscopy confirmed that the delivered DNA was localized in the nuclei (Figures 2 and S2), and a time-resolved membrane leakage experiment and the comparable line widths between the *in vitro* and *in-cell* NMR spectra indicated that this DNA was freely tumbling (Figures S3 and S4). Most importantly, the imino region of the NMR spectrum of a suspension of nontransfected cells showed no signals (Figures 2 and S5, Note S1), whereas NMR spectra of suspensions of cells transfected with DNA–ligand complexes displayed signals originating from the introduced DNA (Figures 2 and S2). The concentration of transfected

complexes in the *in-cell* NMR samples was estimated as 10–15 μ M (Figure S5C). Notably, in all cases, DNA leakage from the cells was negligible, as demonstrated by the imino region of the NMR spectra of the extracellular fluid collected from the samples after the acquisition of *in-cell* NMR spectra (Figures 2 and S2).

The *in-cell* NMR spectra showed well-resolved signal patterns, which were compared with the *in vitro* NMR spectra of unbound DNA and DNA–ligand complexes. For the MH-DNA:netropsin, TT-DNA:1, and TT-DNA:2 complexes, their *in-cell* NMR spectra corresponded to a superposition of the spectra of the ligand-bound and unbound DNA substrates (Figures 2, 3, and S2). However, for the TT-DNA:3 system, the *in-cell* NMR spectrum closely resembled that of the unbound DNA target (Figures 3, S2, and S6). Overall, the *in-cell* NMR data for the TT-DNA:ligand system suggested that, in contrast to DNA–ligand complexes under *in vitro* conditions, complexes in the presence of intracellular factors dissociated to an extent that was ligand dependent. To assess whether the differences in the dissociation level between the TT-DNA complexes of the three naphthalenophanes could be a consequence of their different efficiencies of delivery into cells or whether the *in-cell* NMR spectra instead reflected real differences in the capacity of the individual ligands to bind TT-DNA in the cellular environment, we employed three separate approaches. First, a mock electroporation experiment on the preformed TT-DNA:ligand complexes dissolved in EC buffer was performed: The application of high-voltage pulses (emulating the setup used for electroporation) did not affect the integrity of the individual complexes (Figure S7). Then, we delivered the DNA–ligand complexes into isolated intact nuclei while omitting the electroporation step. Notably, the NMR spectra of the complexes demonstrated only minor dissociation of the TT-DNA:1 complex; however, moderate and dramatic dissociation was observed in the nuclei containing TT-DNA:2 and TT-DNA:3 (Figure 3). The pattern of imino signals in the NMR spectrum of the TT-DNA:3 system in intact nuclei was essentially identical to that of the *in-cell* NMR spectrum of ligand-free TT-DNA (Figure 3 and S6). These observations suggest that nuclear components are potential competitors for ligands 2 and, particularly, 3. To elaborate on this hypothesis, we acquired NMR spectra of TT-DNA:ligand complexes in the presence of calf thymus (ct) DNA to emulate the influence of genomic DNA. The presence of ctDNA resulted in a shift in TT-DNA from its ligand-bound form toward the unbound form, which was particularly pronounced for the TT-DNA:3 complex (Figure S8). These results suggest that dissociation of the complex observed in the intact cells and intact cell nuclei can, at least partly, be attributed to ligand binding to alternative

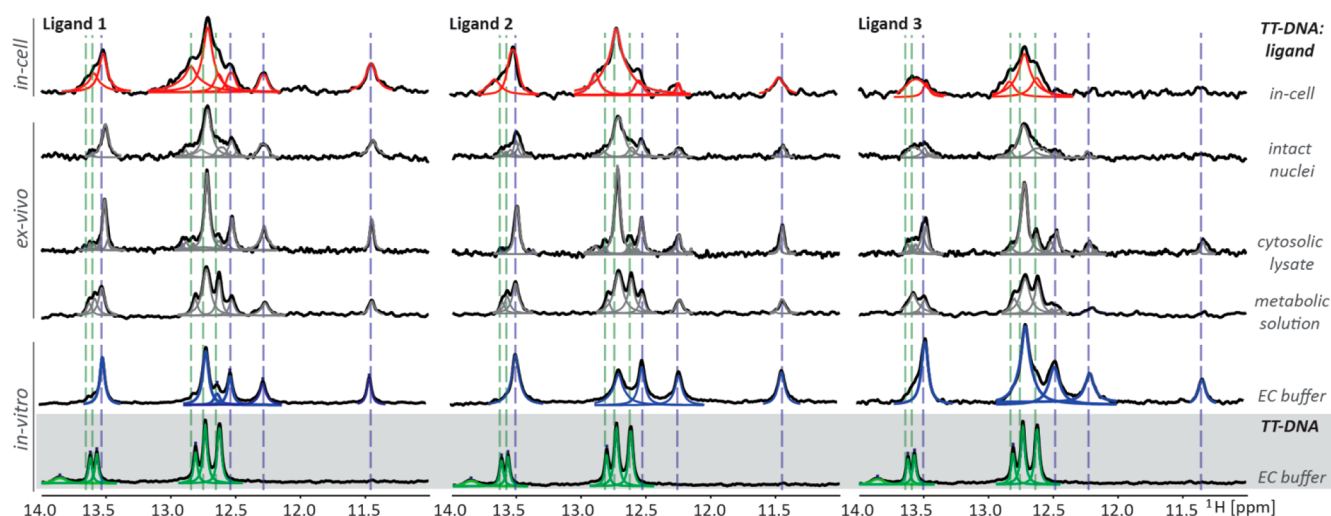


Figure 3. Deconvoluted imino regions of 1D ^1H NMR spectra of TT-DNA in EC buffer and the TT-DNA:ligand complexes *in-cell*, *ex vivo* (intact nuclei/cytosolic lysate/metabolic solution), and in EC buffer (*in vitro*). The vertical green and blue dashed lines mark imino signals specific to the unbound and ligand bound forms of TT-DNA, respectively. For controls of the *in-cell* NMR measurements, see Figure S2.

sites in the genomic DNA, which is in line with the properties of naphthalenophanes previously observed *in vitro*. In fact, while the three ligands share similar physicochemical properties, ligand 3, which has a different topology, nonspecifically binds to double-stranded DNA much more strongly than do ligands 1 and 2.^{16,18} Finally, we recorded NMR spectra of individual complexes dissolved in cytosolic lysate and in a solution of cellular metabolites, corresponding to the low-molecular-weight cellular fraction prepared from the acetonitrile extract of nontransfected cells (Supporting Information, Material and Methods). The acquired spectra of the cytosolic lysate were dominated by signals specific to the formed complexes (Figure 3); however, low-intensity imino signals from unbound TT-DNA were also present, suggesting that some components in the cytosolic environment slightly impact the integrity of individual complexes.

Remarkably, the most pronounced changes in the spectral patterns of all the complexes were observed in the presence of cellular metabolites (Figure 3). While the spectra of the TT-DNA:1 and TT-DNA:2 complexes in the metabolite solution showed signals of comparable intensity from both unbound and ligand-bound TT-DNA, the corresponding NMR spectrum of the TT-DNA:3 complex was dominated by signals from unbound TT-DNA (Figure 3). The latter observation suggests substantial dissociation of the TT-DNA:3 complex in the presence of metabolites. The capacity of metabolites to act as off-targets for ligands was subsequently confirmed using an NMR titration experiment, in which the metabolic solution was titrated with individual ligands at various concentrations (50 and 100 μM). Ligand- and concentration-dependent changes in the positions and intensities of NMR signals from several metabolites, such as ATP, cAMP, GMP, and fumarate, were indicative of ligand–metabolite binding (Figure S9). Accordingly, *in vitro* NMR spectra of 1:1 mixtures of ligands with these metabolites confirmed their capacity to interact with the ligands (Figure S10). Altogether, the *ex vivo* NMR measurements showed that the presence of both metabolic and nuclear components might promote the dissociation of TT-DNA:ligand complexes; however, the extent of this effect was ligand dependent.

To summarize, we demonstrated that DNA–ligand complexes can be monitored in the nuclei of living human cells using NMR spectroscopy. Analysis of *in-cell* NMR spectra using the spectral fingerprint concept¹⁹ reveals whether—and to what extent—the intracellular components and the environment of living cells influence the stability of these complexes. For the TT-DNA:ligand model systems, we identified the nature of the cellular components susceptible to act as off-targets and disrupt the DNA–ligand complexes and confirmed that the dissociation of individual complexes evidenced by *in-cell* NMR spectra was primarily due to the influence of these factors and not an artifact of the electroporation process.

The *in-cell* NMR measurement can be considered a sophisticated competitive binding experiment performed under physiological conditions, where the model target DNA inserted into cells in excess competes for the ligand with everything else in the cell and where the stabilities of DNA:ligand complexes are affected by nonspecific intracellular environmental factors such as crowding, water activity, dielectric permittivity, or local ionic concentration. These conditions are specific to the intracellular environment of intact cells: They are not replicated in cellular lysates/fractions, and they cannot be reliably emulated *in vitro*, as illustrated by the conflicting results obtained from measurements of the TT-DNA:1, 2, and 3 complexes in the presence of either PEG200 or Ficoll 70, most commonly used mimics of molecular crowding^{20–23} and conditions of reduced dielectric permittivity in the intracellular space (Figure S11).^{24–26} In that respect, the *in-cell* NMR experiments are particularly suited for validation of ligands that, after being identified as promising candidates by *in vitro* screening assays, maintain or even reinforce their ability to bind to the target DNA in cells, thereby justifying their further development.

As with other currently available approaches for high-resolution structural analysis of DNA, the presented method provides only information on the behavior of short DNA fragments. In other words, the *in-cell* NMR data do not reflect the influence of chromatin context or packing. However, the fact that DNA-binding ligands generally do not act on closed chromatin but rather during processes involving its open state,

such as transcription and replication, provides justification for the use of our system as a model replicating the effect of DNA-targeting ligands *in vivo*. Furthermore, the presented approach can be directly extended to RNA-based targets. The approach is also invariant of isotopic or chemical labeling of the sample and can be directly adapted for analysis of RNA/DNA:ligand complexes using *in-cell* EPR^{27–29} and *in-cell* single particle FRET.³⁰

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b03031.

Experimental Section, material and methods, Figures S1–S11, and Note S1 (PDF)

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Notes

The authors declare no competing financial interest.

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