Monitoring DNA-Ligand Interactions in Living Human Cells Using High-Resolution NMR Spectroscopy

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Monitoring DNA-ligand interactions in living human cells using high-resolution NMR spectroscopy.

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ABSTRACT: High-resolution studies of DNA–ligand interactions in the cellular environment are problematic due to the lack of suitable biophysical tools. To address this issue, 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 high-resolution NMR data of cells electroporated with pre-formed DNA-ligand complex. The impact of the intracellular environment on the integrity of the complex is assessed on the basis of in-cell NMR signals from unbound and ligand-bound forms of a given DNA target. By using this technique, we studied complexes of model DNA fragments and four ligands, representative of DNA minor-groove binders (netropsin) or ligands binding to DNA pairing defects (naphthalenophanes). We demonstrate that some of the in vitro validated ligands retain their ability to form stable on-target DNA interactions in situ, while other lose this ability due to off-target interactions with genomic DNA as well as cellular metabolic components. Collectively, our data suggest that direct evaluation of behavior of drug-like molecules in the intracellular environment provides important insights for the design and development of DNA-binding ligands with the desired biological action and minimal side effects resulting from off-target binding.

Introduction

DNA is an important target for drug design and development.1 Thus far, the development of DNA-binding ligands has generally relied on data from biophysical assays in vitro, from biological assays in vivo or in cellulo, or from in silico structure-based DNA-targeting strategies. While biological assays have become efficient tools for evaluating the overall biological efficacy of ligands, their principal disadvantage is the difficulty to connect ligand-induced biological phenotypes to the direct action of the ligand on the desired DNA target. In contrast, both in vitro and in silico approaches, which are based on the quantitative characterization of a two-component (DNA-ligand) system under nonnative environmental conditions, allow the detailed characterization of the DNA-ligand complex either in terms of the complex structure or quantitative parameters such as the ligand binding affinity and the thermodynamic stability of the complex using a variety of established biophysical/computational tools.2–10 However, both in vitro and in silico approaches provide information on DNA-ligand interactions in the absence of the complex cellular environment. Consequently, properties of ligands determined from data collected under in vitro and in silico conditions do not automatically translate to the efficacy that the ligands display in vivo, where the presence of alternative drug targets and various environmental factors can influence the formation or stability of DNA-ligand complexes. Undoubtedly, the development of novel tools that will allow both the direct evaluation of drug action under the complex conditions in living human cells and bridge the conventional in vitro and in vivo characterization of DNA-binding ligands is of general interest.

The concept of in-cell NMR spectroscopy of nucleic acids (NASs) in microinjected Xenopus laevis oocytes11 suggest the possibility of studying the structure of DNA drug targets at atomic resolution in the complex environment of living eukaryotic (amphibian) cells. However, in addition to the inherent disadvantage of X. laevis oocytes not being reflective of human physiology, attempts to exploit X. laevis-based techniques for the characterization and monitoring of DNA-ligand interactions have thus far failed to provide in-cell NMR spectra of a quality sufficient for interpretation.12 In 2018, two novel methods for delivering nucleic acid-based targets into living human cells in the quantities required for (in-cell) NMR-based detection were reported.13,14 The method by Yamaoki et al.,13 representing an adaptation of the approach developed by Ogino et al.,15 is based...
on the delivery of nucleic acid-based targets dissolved in nutrient-supplemented cytosolic extract into the intracellular space of living human cells via resealable pores induced by bacterial toxins. In contrast, the method by Dzatko et al.,14 representing an extension of the method introduced by Theillet et al.,16 involved the delivery of nucleic acid targets from a buffered salt solution into the intracellular space of living cells via transiently formed pores that form when cells are exposed to an electric field. Here, we elaborate on the latter (electroporation-based) concept to extend this approach to the characterization of DNA-ligand interactions in the nuclei of living human cells. Using complexes between DNA and netropsin (Figure 1), a polyamide ligand that is representative of compounds that bind to the minor groove of DNA,17-19 and a set of substituted naphthalenophanes (Figure 1), which are representative of DNA base-pairing defects targeting drugs,20,21 as model systems (Explanatory Note S1), we demonstrate that preformed DNA-ligand complexes can be delivered to human cells at amounts sufficient for in-cell NMR detection without compromising the viability of the cells. The resolution of the resulting in-cell NMR spectra is sufficient for discriminating the NMR signals of the bound and unbound forms of the DNA target, which is a critical prerequisite for quantitatively assessing the integrity of the complexes in cells. We show that the vast majority of the introduced DNA is freely tumbling in the intracellular space and is localized inside the cell nucleus. In parallel, we critically evaluate the interference of the electroporation process on integrity of the complexes. We demonstrate a top-down approach, in which in-cell NMR is complemented by ex vivo NMR measurements of cellular extracts to facilitate the identification of the ligand(s)’ cellular off-targets. Altogether, we show for the first time that DNA-ligand interactions can be characterized in the nuclei of living human cells at atomic resolution. Our data highlight the potential of this method for the quantitative characterization of the stability of DNA-ligand complexes in vitro, which is inherently distinct from what can be achieved with commonly used in vitro models.

Results

Factors limiting the delivery of the ligand and its DNA target into the cellular interior via electroporation. Using in-cell NMR spectroscopy to assess the influence of intracellular environmental factors on the interactions between a given DNA target and a ligand (drug-like molecule) presumes that both the target and ligand can be delivered into the cells in a way that does not compromise cell viability and results in the intranuclear localization of the delivered DNA and ligand. In addition, the ligand and the DNA need to be delivered into cells in sufficient amounts to allow NMR detection, and when using this technique to compare the capacities of distinct ligands to bind single DNA targets, the delivery must be achieved in a way that eliminates potential differences in the uptake of various ligands by the cells. The latter two conditions preclude employing the spontaneous uptake of the DNA/ligand and require the engagement of an invasive approach. Regardless of the nature of the invasive approach, there are two conceptually distinct methods of target/ligand delivery into cells. The target and the ligand can be either delivered independently of each other (in two successive steps) or the target and the ligand can be delivered in a single step as a preformed DNA:ligand complex.

To evaluate the separate delivery of a ligand and DNA target into cells using electroporation, two experimental setups employing the naphthalenophane 2,7-BisNP-NH, which is representative of DNA mismatch-site binding ligands,20 and a T-T mismatch-containing DNA (TT-DNA) substrate21 as a model system were tested (Figure 1). In the first set-up, herein referred to as the “D/L experiment” (for “DNA then ligand”), the cells were transfected with the (FAM)-TT-DNA target, and following a 1 hour recovery period, they were then transfected with the ligand. In the second setup, herein referred to as the “L/D experiment” (for “ligand then DNA”), the ligand was transfected first, and the (FAM)-TT-DNA was transfected after the recovery period. To evaluate the possibility of delivering the (FAM)-TT-DNA and ligand in a single transfection step, herein referred to as the “D+L experiment” (for “DNA and ligand”), the preformed (FAM)-TT-DNA:2,7-BisNP-NH complex was electroporated into the cells. Illustrative results from flow cytometry (FCM), which indicate the cell viability after transfection as well as the efficiency of the transfection step, and images from confocal microscopy, which indicate the intranuclear localization of the delivered DNA from the individual transfections, are displayed in Figure 2.

In all the experiments (D/L, L/D, and D+L), the great majority of transfected (FAM)-TT-DNA was localized in the cell nucleus (Figure 2A). Compared to the D+L experiment, which involved a single transfection step and resulted in ~ 92.1% of the cells remaining viable, the cell viability in the experiments that used two consecutive electroporation steps was notably reduced (Figure 2B, Figure S1 – Supporting Information). The outcome of the “mock” electroporation experiment indicated that the viability of the cells was adversely influenced primarily by the second electroporation step. After the first control electroporation (no ligand/no DNA), the FCM plot indicated ~ 88.1% of the cells were viable, and only ~ 60.2% of the cells were viable following the second (no ligand/no DNA) electroporation.
In the second round of electroporation pulses. Taken together, nuclei (and the cells) upon disruption of the cell membrane by spontaneous diffusion of the DNA from cell nuclei (and the cells) into the nuclei was localized into the nuclei. The introduced DNA was devoid of (FAM) fluorescent (TT DNA) and the cells met the qualitative criteria required for the preparation of an in-cell NMR sample; its field NMR spectrometers and cryogenic probe heads, its timely analysis was (~ 5 µM. The D+L experiment described above demonstrated that the cotransfection of the preformed complex preserves the viability of cells and leads to intranuclear localization of the delivered DNA. However, this experiment provides no information on whether the DNA/ligand complex was delivered into cells in an amount sufficient for (in-cell) NMR detection. To address this issue and to test the generality of the cotransfection approach, we attempted preparative scale (in-cell NMR) transfections of two model preformed DNA-ligand complexes: i) the TT-DNA:2,7-BisNP-NH complex (see above) and ii) the complex between netropsin, a polyamide ligand that is representative of compounds that bind to the minor groove of DNA, and its model target, hairpin-based DNA (MH-DNA) (Figure 1). Both (FAM)-MH-DNA:netropsin and (FAM)-TT-DNA:2,7-BisNP-NH complexes, pre-formed in EC buffer, were separately introduced into ~ 1.3x10^6 HeLa cells.

As evidenced from the confocal microscopy images (A) and double-staining (PI/FAM) FCM analysis (B) of HeLa cells electroporated with either a “mock” (EC buffer) or (FAM)-TT-DNA and 2,7-BisNP-NH in a form of pre-formed complex (in single electroporation step (D+L) or separately in two discrete electroporation steps (D/L and L/D). In the confocal images, the green color marks the localization of (FAM)-TT-DNA, while the blue color marks cell nuclei stained by Hoechst 33342. In the FCM plots, percentages of viable non-transfected cells, viable TT-DNA containing cells, non-transfected dead/compromised cells, and dead/compromised cells transfected with the TT-DNA are indicated in the bottom left, bottom right, top left, and top right quadrants, respectively. For the extended version of Figure 2 - see Figure S1 (Supporting Information).

Figure 2. Confocal microscopy images (A) and double-staining (PI/FAM) FCM analysis (B) of HeLa cells electroporated with either a “mock” (EC buffer) or (FAM)-TT-DNA and 2,7-BisNP-NH in a form of pre-formed complex (in single electroporation step (D+L) or separately in two discrete electroporation steps (D/L and L/D). In the confocal images, the green color marks the localization of (FAM)-TT-DNA, while the blue color marks cell nuclei stained by Hoechst 33342. In the FCM plots, percentages of viable non-transfected cells, viable TT-DNA containing cells, non-transfected dead/compromised cells, and dead/compromised cells transfected with the TT-DNA are indicated in the bottom left, bottom right, top left, and top right quadrants, respectively. For the extended version of Figure 2 - see Figure S1 (Supporting Information).
cell NMR spectra of the TT-DNA:2,7-BisNP-NH complex and in the reference in vitro spectra (Figure S3C – Supporting Information). Notably, in both cases, the leakage of (complexed) DNA from the cells was negligible, as demonstrated by the imino region of the 1D 1H NMR spectra of the extracellular fluid taken from the in-cell NMR samples after the acquisition of the in-cell NMR spectra (Figure 3C,F). The line-widths of the imino signals in the in-cell NMR spectra were only slightly greater than those of the corresponding in vitro spectra (Figure 3C,F and Figure S4 – Supporting Information). This increase can be attributed to the inherent inhomogeneity of the in-cell NMR samples and the viscosity of the intracellular environment. Notably, the comparable line-widths of the imino signals from the buffered solutions (in vitro) and in the in-cell NMR spectra unambiguously indicated that the in-cell NMR signals correspond to the freely moving DNA:ligand complexes. Any interactions with (> 15 kDa) proteins (or other high-molecular-weight molecule(s)) are expected to cause significant broadening of the in-cell NMR signals due to increased correlation times and exchange processes.24,25 At a given intracellular DNA:ligand complex concentration, such NMR signals would fall below the detection limit. Though, the presence of the complexes’ specific spectral signatures in the in-cell NMR spectra acquired on the transfected cells confirmed the presence of the complexes in the intracellular space, in both cases, the in-cell NMR spectra of the complexes were different from those of their in vitro counterparts (Figure 3C,F). The spectral differences are discussed below.

In-cell NMR signals are of nuclear origin. As previously mentioned, the confocal microscopy images of the transfected cells indicated the nuclear localization of the transfected (FAM)-TT/MH-DNA. However, these confocal microscopy images cannot be used to distinguish between DNA immobilized by cellular factors and freely tumbling DNA, while (in-cell) NMR spectroscopy only shows signals from freely tumbling DNA. Therefore, one could argue that whereas the majority of the observed fluorescence in the nuclear foci corresponds to immobilized (FAM)-TT/MH-DNA associated with nuclear components such as proteins and/or genomic DNA, the signals observed in the in-cell NMR spectra might originate from the residual freely tumbling TT/MH-DNA uniformly distributed in the intracellular space, i.e., throughout the nucleus and cytosol. To estimate the ratio between the immobilized and freely moving DNA in the nuclear foci, (FAM)-TT-DNA could be visualized in the transfected cells treated with the lysis buffer when the fluorescence detection threshold was increased (Figure 4). These observations suggest that the vast majority of (FAM)-TT-DNA (over 92%) that was localized in the nucleus according to confocal microscopy was freely tumbling in the intranuclear space and thus could be detected by NMR spectroscopy (Figure 4B, Figure S5 – Supporting Information). At the same time, this observation indicates that the spontaneous localization of short DNA fragments into the cell nucleus is dependent (due to unknown mechanism) on an intact cell membrane. These results provide an explanation for the loss of the (FAM)-TT-DNA fluorescence signal from the transfected cells upon application of the second, cell membrane-disrupting electroporation pulse in the D/L experiment (described above, cf. Figure 2).

In-cell NMR spectra are indicative of partial dissociation of the complexes in the intracellular space. As previously mentioned, the in-cell NMR spectra of the TT-DNA:2,7-BisNP-NH and MH-DNA:netropsin complexes were not identical to those of their respective in vitro counterparts. More specifically, in

![Figure 3](image-url)
vitro conditions, both complexes might partially dissociate in the presence of intracellular environmental factors.

To assess the reliability of the in-cell NMR measurements for determining the relative capacities of different ligands to form complexes with a single DNA target under physiologically relevant conditions, we assessed the in-cell NMR data of the TT-DNA:2,7-BisNP-NH complex in conjunction with data for complexes between TT-DNA and two other TT-DNA-binding ligands, namely, 2,7-BisNP-O and 1,5-BisNP-O (cf. Figure 1B). While these two ligands and 2,7-BisNP-NH share the same naphthalene scaffold, their capacities to bind and stabilize TT-DNA in dilute buffer solutions and in the presence of calf thymus (ct)DNA, a mimic of the off-target sites in genomic DNA, are distinct. The preformed complexes between TT-DNA and 2,7-BisNP-O and 1,5-BisNP-O were separately introduced into living HeLa cells using a procedure analogous to that employed for the TT-DNA:2,7-BisNP-NH complex. Similar to what was observed with the TT-DNA:2,7-BisNP-NH complex, the transfections of both the TT-DNA:2,7-BisNP-O and TT-DNA:1,5-BisNP-O complexes into the HeLa cells were highly effective; (~ 94.7% efficiency in both cases) (Figure 5A,D). Importantly, out of cells transfected with the TT-DNA:2,7-BisNP-O and TT-DNA:1,5-BisNP-O complexes, i.e., the cells potentially contributing to the in-cell NMR signals, ~ 98.6 and ~ 98.3%, respectively, remained viable (Figure 5A,D). The confocal microscopy images confirmed the delivered DNA was localized in the nuclei (Figure 5B,E). Notably, similar to the imino region of the deconvoluted 1D 'H in-cell NMR spectrum of the TT-DNA:2,7-BisNP-NH (Figure 3F), the deconvoluted imino regions of the in-cell NMR spectra indicated that the complexes dissociated in the intracellular space (Figure 5C,F). However, while the in-cell NMR spectra of the TT-DNA:2,7-BisNP-NH (Figure 3F) and TT-DNA:2,7-BisNP-O (Figure 5C) complexes were indicative of partial dissociation, the in-cell NMR spectrum of the TT-DNA:1,5-BisNP-O complex showed no complex-specific signals, and its overall pattern was essentially identical to that of the in-cell NMR spectrum of the cells transfected with TT-DNA alone (Figure 5F, Figure S6 – Supporting Information). These observations indicate the absence 1,5-BisNP-O-bound TT-DNA in the intracellular space and suggest that the complex is fully (or mostly) dissociated in the intracellular space.

However, the comparative interpretation of the in-cell NMR spectra in terms of the bound and unbound TT-DNA is only possible under the assumption that the individual ligands/DNA complexes were delivered into the intracellular space under essentially identical concentrations (i.e., with similar efficiencies). The alternative explanation of the in-cell NMR spectra of cells transfected with the pre-formed TT-DNA:1,5-BisNP-O complex, which is essentially the same as the in-cell NMR spectrum of ligand-free TT-DNA (Figure S6 – Supporting Information), might simply indicate that 1,5-BisNP-O entered the cell at a much lower concentration than the DNA target (TT-DNA) or that it did not enter the cell at all.

Electroporation might interfere with the chemical equilibria of systems with notably different unfolding-folding rates. To explore the possibility of complex dissociation during electroporation, we first performed a mock electroporation experiment with the TT-DNA:2,7-BisNP-NH, TT-DNA:2,7-BisNP-O and TT-DNA:1,5-BisNP-O complexes in EC buffer followed by immediate acquisition of the NMR spectra. In all cases, the resulting NMR spectra were essentially identical to those acquired prior to the electroporation treatment (Figure 6A, Figure S7 – Supporting Information). These results suggest that the electroporation either does not perturb the complexes and/or that the perturbation is fully reversible. To clarify this issue, we performed an analogous experiment using a 5'-d(GTGTGGGTTG)-3' oligonucleotide with a so-called G-hairpin structure. This structure has a notably longer folding time relative to its unfolding time. On the time scale of the indicated experiment (10 min), the unfolding/folding of the G-hairpin can be considered essentially irreversible, as evidenced by the in vitro NMR spectra of the G-hairpin acquired prior to heating and after ~ 10 min of heating at 95 °C. While prior to heating at 95 °C, the imino region of the 1D 'H NMR spectrum of the G-hairpin sample displays signals typical of the G-hairpin structure, the imino region of the spectrum acquired after 10 min of thermal unfolding shows no imino signals, which indicates the absence of folded G-hairpin structures in the sample (Figure 6B). These data demonstrate that any induced early unfolding event (at time scales between 0-10 min) should be indicated by clear reductions in the intensities of the imino signals (which are specific to folded species) in the NMR spectrum. To evaluate the impact of the composite pulse sequence (100 μs/1000 V – 5 s delay – 30 ms/350 V) employed for DNA complex electroporation, the G-hairpin sample was subjected to “mock” electroporation in the EC buffer. As shown in Figure 6C, the imino NMR signals from the G-hairpin sample subjected to the mock electroporation were approximately 32% less intense than those of the reference (nonelectroporated) sample. These observations indicate that the electroporation process employed in the preparation of in-cell NMR samples might partially perturb the integrity of molecular systems with notably different unfolding – folding/association – dissociation rates.

To explore whether the presence of unbound TT-DNA in NMR spectra of the cells transfected with the individual complexes was a consequence of differences in the efficiencies of the deliveries of the individual ligands into cells or whether the in-cell NMR spectra reflect real differences in the capacities of the individual ligands to bind TT-DNA in the cellular environment, we analyzed the NMR spectra of the individual complexes in the cytosolic and metabolic cellular extracts (lysates) and in isolated intact cell nuclei, i.e., under conditions herein collectively referred to as ex vivo conditions. While cellular extracts/intact nuclei undoubtedly represent nonnatural and highly

![Figure 4](image-url)  
**Figure 4.** A) Overexposed confocal microscopy images of cells transfected with (FAM)-TT-DNA (in green) acquired prior (LEFT) and 2 minutes post exposure (RIGHT) to the cell membrane lysis buffer (Buffer A – cf. Supporting Information and Methods). B) Column plot: Quantitative processing of the microscopic images in terms of total intracellularly localized (FAM)-TT-DNA and its fraction stably associated with cellular components.
manipulated complex solutions, they simulate many factors present in the intracellular space; as a first approximation, isolated intact nuclei can be considered a model environment mimicking the presence of genomic DNA and nuclear proteins. The cytosolic lysate can be considered a dilute model of the cell cytosol environment, as it accounts for the presence of cytosolic components such as small RNAs, proteins, and metabolites as well as nuclear components that are able to cross intact nuclear membranes. The metabolic extract, unlike the cytosolic fraction, provides an environment containing undiluted low-molecular-weight compounds present in the intracellular environment. Importantly, in contrast to the intact cells, the preformed DNA:ligand complexes can be quantitatively and in a controlled manner delivered (by a simple addition into the extract) prior to NMR.

To address potential biases in the in-cell NMR measurements from differences in the uptake efficiencies of the individual ligands by the cells in the course of electroporation, the preformed TT-DNA:2,7-BisNP-NH, TT-DNA:2,7-BisNP-O, and TT-DNA:1,5-BisNP-O complexes were separately mixed into the individual ex vivo fractions to reach the final complex concentrations of 50 µM. The resulting mixtures were evaluated by NMR spectroscopy using the same parameters as those employed for the acquisition of the in-cell NMR spectra. The resulting ex vivo NMR spectra, along with essential in vitro controls, including the NMR spectra of the complexes in the buffers employed for the preparations of the extracts, are displayed in Figure 7. The NMR spectra of the individual TT-DNA:ligand complexes acquired in the cytosolic lysate were dominated by the signals specific to the formed complexes (Figure 7). However, low-intensity imino signals from the unbound TT-DNA were also present in the spectra, suggesting that the components in the cytosolic environment impact the integrity of individual complexes to a small extent. While a minute dissociation of the complex was also indicated by the ex vivo NMR spectrum of the intact nuclei for the TT-DNA:2,7-BisNP-NH, moderate and dramatic dissociation of the complex were observed in the NMR spectra of the intact nuclei solutions of the TT-DNA:2,7-BisNP-O and TT-DNA:1,5-BisNP-O, respectively (Figure 7). Notably, the pattern of imino signals in the NMR spectrum of the TT-DNA:1,5-BisNP-O complex in the intact nuclei solution was essentially identical to that of the (in-cell) NMR spectrum of ligand-free TT-DNA (Figure 7, Figure S6 – Supporting Information). These observations suggest that nucleic component(s) is/are a potentially important cellular off-target for both 2,7-BisNP-O and 1,5-BisNP-O, particularly for 1,5-BisNP-O (see below). In general, the most pronounced changes in the spectral patterns of all the complexes were observed in the presence of cellular metabolites (Figure 7). While the ex vivo NMR spectra of the TT-DNA:2,7-BisNP-NH and TT-DNA:2,7-BisNP-O complexes in the metabolic extract showed signals of comparable intensity from both the unbound and ligand-bound TT-DNA, the corresponding ex vivo NMR spectrum of the TT-DNA:1,5-BisNP-O complex was dominated by signals from unbound TT-DNA (Figure 7, Figure S6 – Supporting Information). The latter observation suggests substantial dissociation of the TT-DNA:1,5-BisNP-O complex in the presence of metabolic components.

Altogether, the ex vivo NMR measurements showed that the presence of both metabolic and nuclear components might promote the dissociation of the TT-DNA:ligand complexes (see be

![Figure 5. Double-staining (PL/FAM) FCM analysis (A) and confocal microscopy images (B) of cells co-transfected with (FAM)-TT-DNA:2,7-BisNP-O 1:1 complex. C) Deconvoluted Imino regions of 1D 1H NMR spectra of the TT-DNA in vitro (black/green), and TT-DNA:2,7-BisNP-O 1:1 complex in vitro (black/blue) and in cell (black/red). Imino region of 1D 1H NMR spectrum of extracellular fluid taken from the in-cell NMR sample after in-cell spectrum acquisition (gray). D, E) and F) analogous data for the TT-DNA:1,5-BisNP-O 1:1 complex. Spectra in black and green/blue/red are representative of raw and deconvoluted NMR spectra, respectively. Vertical dashed green and blue lines marks imino signals specific for the unbound and a ligand bound form of given DNA target. (comparison). However, the extent of the effect appears to be ligand dependent. Importantly, qualitative agreement between the behavior of the individual complexes under in-cell and ex vivo conditions confirmed that the dissociation of the individual complexes observed in the in-cell NMR spectra was primarily due to the influence of intracellular factors and not an artifact of the electroporation process.

Top down ex vivo NMR analysis to help identify the ligands’ cellular off-targets. The spectra of the TT-DNA:ligand complexes in the presence of low-molecular-weight (LMW) compounds (metabolites) and those acquired in intact cell nuclei (Figure 7) indicated that cellular metabolites and nuclear components might be direct off-targets of the ligands. However, living cells cannot be readily manipulated to determine the factors affecting integrity of the complexes in cells. Unlike living cells, the cellular extract can be manipulated in a controlled manner. At the same time, fractionalizing the cellular content into less complex cellular extracts suggest the possibility of identification of factors that affect behavior of DNA-ligand complexes in
cells. Considering these two factors, we attempted to use ex vivo NMR analysis to identify the cellular off-targets of the ligands.

To directly confirm that cellular metabolic components can act as off-targets, the LMW cellular fraction prepared from the acetonitrile extract of nontransfected cells was titrated with the individual ligands at various concentrations (50 and 200 µM) (Figure S8 – Supporting Information). As shown in Figure S8B, no signals corresponding to the free ligands were observed in the samples consisting of the LMW cellular fraction even at a ligand concentration of 200 µM. However, the positions and intensities of the NMR signals from a number of metabolic components were shifted and/or reduced, respectively, in the presence of the ligands, and these shifts/reductions were ligand-concentration dependent (Figure S8C - Supporting Information), indicating the corresponding metabolic components are potential binders of the ligands (cellular off-targets). Specifically, pronounced ligand concentration-dependent decreases in the intensities and chemical shift changes of the NMR signals characteristic of the aromatic (H8) protons in nucleotides such as ATP, ADP, (c)AMP or GMP were observed (Figure S8C - Supporting Information). A moderate ligand dose-dependent reduction in signal intensity was also observed for the signal at ~6.5 ppm (Figure S8C - Supporting Information), which corresponds to fumarate.28 To probe the capacity of the metabolites to interact with the ligand, 1D 1H NMR spectra of a 1:1 mixture of the ligands and the metabolites suggested by the titration experiment were acquired (Figure S9 – Supporting Information). Notable differences between the spectra of 2,7-BisNP-NH and 2,7-BisNP-NH in the presence of the indicated metabolites confirmed the existence of direct interactions between this ligand and the individual metabolic components. Notably, for ATP, GMP, and fumarate, this interpretation is consistent with in vitro data reported for the related naphthalenophane compound.29 Analogous experiments with 2,7-BisNP-O showed that essentially the same set of metabolites can also interact with this ligand (Figure S9 - Supporting Information). Although the ex vivo NMR spectrum indicated almost complete dissociation of the TT-DNA:1,5-BisNP-O complex in the metabolic fraction (Figure 7), the ligand dose-dependent changes in both metabolic signals’ positions and intensities decrease observed in the titration experiment were rather small (Figure S8C - Supplementary Information). This observation might suggest that 1,5-BisNP-O is more promiscuous than 2,7-BisNP-NH/O, i.e., binding to a notably wider variety of metabolic components (in this case, the changes in the levels of individual interacting metabolites would be small and undetectable by NMR spectroscopy). To elaborate on the observed interference to the integrity of individual complexes from nuclear components (see above), we acquired spectra of the TT-DNA:ligand complexes in the presence of the calf thymus (ct) DNA to emulate influence of genomic DNA. As shown in Figure S10 (Supporting Information), the presence of ctDNA resulted in shift between ligand bound and unbound forms of TT-DNA towards the unbound form, particularly in the case of the TT-DNA:1,5-BisNP-O complex. These data are suggestive that at least a part of the in-cell NMR phenotype (the complex dissociation) observed in the intact cell nuclei can be attributed to the ligand binding to the alternative (off-targets) targets in the genomic DNA.

While a complete identification of the metabolic components and a precise determination of the genomic ligand off-target sites that can bind the naphthalenophanes goes beyond the scope of the present study, the performed analysis clearly demonstrates that by employing a top-down approach, the

Figure 6. A) Imino regions of 1D 1H NMR spectra of 2,7-BisNP-NH:TT-DNA 1:1 complex in the EC buffer acquired prior (black) and immediately after application (red) of high (1000 V/100 µs) and low (350V/30 ms) voltage pulses separated by 5s delay. B) Imino region of 1D 1H NMR spectrum of the 5'-d(GTGTGGGTGTG)-3' in the EC buffer acquired prior (black) and immediately post thermal unfolding (10 min at 95°C + 10 min cooling step required cool down the sample to 20°C) (red). C) Imino region of 1D 1H NMR spectrum of the 5'-d(GTGTGGGTGTG)-3', forming a G-hairpin structure, in the EC buffer acquired prior (black) and immediately post application of high (1000 V/100 µs) and low (350V/30 ms) voltage pulses separated by 5s delay (red).

in-cell NMR data can facilitate the identification of the cellular off-targets of the ligand.

Non-specific environmental factors can modulate the integrity of the DNA-ligand complexes in the intracellular space. In the intracellular space, in addition to specific (chemical) factors, the DNA-ligand complexes are influenced by physical factors/fields, which are generally poorly understood. Among these environmental parameters, molecular crowding and dielectric permittivity are broadly recognized as environmental parameters that are notably distinct between dilute saline solutions, representing commonly used in vitro conditions for DNA-ligand studies, and the intracellular space.30,31 To assess the potential influence of these two factors on DNA-ligand interactions, we acquired NMR spectra of the TT-DNA:2,7-BisNP-O complex in the absence and presence of either the polyethylene glycol (PEG) 200 or Ficoll 70 (Figure 8), which are commonly used as mimics of molecular crowding31-34 and conditions of reduced dielectric permittivity in intracellular space.30,35,36 In the presence of the 40% of the PEG, the NMR spectra indicate essentially complete complex dissociation (Figure 8); however, in the presence of the 20% Ficoll (providing approximately the same water excluded volume as the 40% PEG200 solution), the NMR spectra of the complex show no signs of the complex dissociation (Figure 8). The data acquired in the presence of the PEG200 indicate that the specific intracellular factors are not that only parameters responsible for the observed in-cell NMR phenotype; the conflicting results obtained from measurements of the PEG and Ficoll solutions only highlight the importance of in-cell NMR data as being physiologically relevant and essential for the reliable interpretation of the structural data acquired under simplistic in vitro conditions. Overall, the NMR data acquired in the presence of synthetic additives (Ficoll, PEG200) as well as the ex vivo NMR data measured in the cellular fractions indicate that there might be additional factors at play in the intracellular space that can influence
Figure 7. (Deconvoluted) Imino regions of 1D $^1$H NMR spectra of the ligand (2,7-BisNP-NH, 2,7-BisNP-O, or 1,5-BisNP-O) bound and unbound TT-DNA in vitro (A) and (C), respectively. B) (Deconvoluted) Imino regions of 1D $^1$H NMR spectra ex vivo (in intact cell nuclei and cytosolic and metabolic cellular extracts), and in-cells. In vitro spectra were acquired either in the EC buffer or buffer A (indicated). Spectra in black and green/blue/red/gray are representative to raw and deconvoluted NMR spectra, respectively. Vertical dashed green and blue lines marks imino signals specific for the unbound and ligand bound form of given DNA target, respectively.

the interactions between the DNA and ligand. Undoubtedly, the measurements in cellular extracts or in synthetic models of the cellular interior might provide important insights into the nature of some of these factors; however, only measurements in intact cells can capture the overall/global impact of the complex cellular environment on the studied DNA-ligand system.

Discussion

Thus far, attempts to extend the concept of in-cell NMR spectroscopy to monitoring NA-ligand interactions in living eukaryotic cells (X. laevis oocytes) have failed to reconstitute the NA-ligand complexes in the intracellular space and/or to provide in-cell NMR spectra of the complexes in sufficient resolution to allow their interpretation. Here, we demonstrated that DNA-ligand complexes can be monitored using NMR spectroscopy using a suspension of living human cells electroporated with a preformed DNA-ligand complex. We demonstrate that the quality/resolution of the resulting in-cell NMR spectra of the complexes allows their interpretation using the spectral fingerprint concept; the deconvolution of the in-cell NMR spectra to and/or simple visual comparison of the in-cell spectra to the in vitro reference spectra of the ligand-bound and unbound forms of the DNA target directly indicate if the intracellular environment of living cells interferes with the complex formation/integrity and to what extent the environment affects these parameters. In all presented cases, the in-cell NMR spectra of the DNA-ligand complexes were distinct from the corresponding in vitro NMR spectra, indicating environmentally induced dissociation of the complexes. The fact that observed degrees of dissociation were ligand-specific highlights the potential application of the presented in-cell NMR-based technique for assessing the relative capacities of various ligands (drugs) to form complexes with given DNA targets under physiological conditions. However, it needs to be stressed that the presented in-cell NMR data only provide semi-quantitative information on the impact of the intracellular environment on the integrity of the complexes. The impacts of specific intracellular factors (such as metabolites) might be underestimated because the concentrations of the delivered material required for NMR-based detection with the current generation the NMR hardware (NMR spectrometers and cryogenic probe heads) could be higher than endogenous levels of certain potential cellular off-targets. Compared to the established in-cell NMR approach based on microinjection into X. laevis oocytes, which requires that the intracellular concentrations of exogenous material be in the range of 150-200 µM, the presented approach operates at concentrations of ~ 10-15 µM, i.e., approximately one order of magnitude lower. Nevertheless, these intracellular concentrations are still above the physiological range (endogenous levels of many of the relevant cellular factors are in the mid-to-high nM range).

It is also important to consider that delivered exogenous material might disturb the parameters of nonspecific cellular environments. Although the mechanisms responsible for maintaining homeostasis of the intracellular space that are active in liv-
ing cells appear to efficiently compensate for potential disturbances in intracellular pH and/or ion levels, the impact of the delivered material on other nonspecific parameters of the intracellular space, e.g., on the level of molecular crowding, is not currently known. Undoubtedly, the magnitude of this problem will be (at least partially) diminished in the near future due to the launching of a new generation of NMR spectrometers operating at an ultrahigh magnetic field (28 Tesla), which will provide improved sensitivity in NMR detection and allow the acquisition of in-cell NMR spectra of exogenous material at lower intracellular concentrations.

The launch (commercial availability) of recently developed $^{19}$F-modified nucleoside-phosphonamidites, particularly those used for the site-specific preparation of CF$_3$-substituted oligonucleotides, will also help minimize this problem. Nevertheless, further developments will be needed to completely eliminate this problem. Current developments in the field of dynamic nuclear polarization (DNP)-enhanced NMR detection, particularly advances in stable spin labels allowing the employment of DNP at room temperature, holds great promise for a notable reduction in the concentration of exogenous material, allowing in-cell NMR measurements with greater physiological relevance.

We showed that the proportion TT-DNA bound to the macrocyclic ligands was not affected by electroporation. However, we did show that electroporation might considerably perturb molecular systems with notable differences between the rates of folding and unfolding. This observation must be considered as another limitation in the application of electroporation for the preparation of in-cell NMR samples, as it provides a plausible explanation for the problems noted in the preparation of in-cell NMR samples of folded proteins. While in-cell NMR samples of intrinsically disordered proteins can generally be prepared using an electroporation-based approach, the method often appears impractical (or notably less efficient) for preparing samples of structured proteins, as their unfolding (even transient unfolding) might make the proteins prone to irreversible aggregation. For systems with notable differences between their rates of folding and unfolding/association and dissociation, alternative means of preparing in-cell NMR samples should thus be considered. However, at the present time, the approach based on reversible pore formation in the cell membrane by the action of the pore-forming toxin, streptolysin O is the only alternative.

While in this case, the formation of the pores does not require the application of an external physical field that could influence the integrity of the complex, the principal disadvantage of this approach is the fact that the delivery of the exogenous material needs to be initiated from cytosolic extract supplemented with “nutrients” such as ATP. This makes this approach unsuitable for cases where either the DNA target or the ligand off-targets cytosolic or metabolic components such as what is seen with the macrocyclic ligands discussed herein.

The monitoring of the DNA-ligand complexes in vivo using in-cell NMR spectroscopy presented here was exclusively (and deliberately) based on the imino signals from the 1D $^1$H (in-cell) NMR spectra acquired from cells transfected with nonlabeled oligonucleotides. In contrast to the observation of cellular proteins using in-cell NMR, which almost always requires the use of isotopically (e.g., $^{13}$C or $^{15}$N)-labeled or covalently modified (e.g., $^{19}$F) material to differentiate the NMR signals of the protein of interest from those of the cellular background, the direct observation of the NMR signals of the imino hydrogens from the exogenous DNA is possible because the imino region of the NMR spectra of eukaryotic cells shows no signals from cellular background (Figure S3, Explanatory Note S2 – Supporting Information). The detection of $^1$H nuclei has two principal advantages. First, considering the low intracellular concentration of the introduced DNA target/complex in intracellular space (~ 10-15 μM), the detection of $^1$H (an NMR-active nucleus with superior sensitivity and 99.98% natural abundance) typically allows the acquisition of a good quality intracellular NMR spectrum in 30-40 min. This acquisition time is shorter than the time frame associated with the induction of cellular metabolic stress due to the lack of nutrients and oxygen in the NMR tube (~ 1-1.5 h). While the problems associated with metabolic stress can be diminished by the use of bioreactors, short acquisition times are still needed to minimize spectral artifacts arising from DNA degradation in the intracellular space.

Second, considering the cost of isotopically labeled or chemically modified DNA, the fact that in-cell NMR spectroscopy can be performed without isotopic labeling/filtering makes the method more economically viable. However, it must be noted that detection of the $^1$H nucleus limits the analysis to DNA/DNA-ligand systems. The integrals (area under the peaks) of the imino signals originating from the bound and unbound complexes are similar or identical. However, this condition is rarely met because the binding of most DNA-

![Figure 8](image)

**Figure 8.** Imino region of the 1D $^1$H NMR spectra of the TT-DNA:2,7-BisNP-O complex in absence and the presence of the PEG200 (A) and Ficoll 70 (B). The spectra were acquired in EC buffer at 20°C. Note: 20% Ficoll provides about the same water exclusion effect as 40% of PEG200.
binding ligands results in a more stable species, and the base-pair opening is inversely correlated with DNA stability. In other words, the populations of ligand-bound and unbound DNA indicated by the integrals of the imino signals in the in-cell NMR spectra will most likely be overestimated and underestimated, respectively. For the same reason, accurate quantitative information on the populations of DNA bound and stabilized by two ligands, which might differentially stabilize the species in the intracellular space, cannot be directly obtained from signals of exchangeable $^1$H nuclei.

While precise, quantitative information on the integrity of the complex cannot be obtained from the imino signals from either the ex vivo or in-cell $^1$H NMR spectra, the spectra still provide semi-quantitative estimate of the extent of complex formation in the intracellular space. For example, the in-cell NMR data indicative of comparable capacities of 2,7-BisNP-NH and 2,7-BisNP-O to bind TT-DNA in cells suggest these two ligands are suitable candidates for drug development. In contrast, 1,5-BisNP-O can be excluded from further consideration in drug development because the in-cell NMR data indicate that its capacity to bind the target (TT-DNA) is essentially lost in the presence of intracellular factors. When precise quantitative interpretation is required (e.g., in the final stage of lead compound identification), the present procedure should be performed using isotopically ($^{13}$C or $^{15}$N) labeled or chemical-modified ($^{19}$F) samples. The recently introduced CF$_3$-based modifications of oligonucleotides appear particularly suitable for quantitative analysis using the techniques discussed herein. In addition, the $^{19}$F chemical shift is sufficiently sensitive for the discrimination of bound/unbound and folded/unfolded DNA; the freely rotating CF$_3$ moiety provides sufficient sensitivity to avoid extended acquisition times while at the same time, providing an in-cell NMR spectra free of signals from the cellular background. However, at the present time, the use of CF$_3$-based modifications for routine applications remains limited due to technical challenges associated with the bulk production of modified precursors and because the respective precursors are not commercially available.

Although the in-cell NMR spectra reflect the behavior of the DNA-ligand complexes under physiologically relevant conditions, the sensitivity of the sample to external factors, the intracellular concentration of the delivered DNA-ligand complex being close to the NMR detection limit and the rather limited time for manipulating living cells in the NMR spectrometer generally prevent obtaining of more detailed information related to the origin of the phenotype observed in cells. As demonstrated here, this limitation can be overcome to a great extent by complementing in-cell NMR data with corresponding data acquired in cellular lysates/extracts. We show that in cases when ex vivo (artificial complex solution) NMR spectra recapitulate the in-cell NMR phenotype (an essential in vivo reference), downstream analysis of the ex vivo NMR data might facilitate the identification of the specific cellular component(s) acting as the ligand(s’ cell-off-target(s).

As with other currently available approaches for high-resolution structural analysis of nucleic acids (X-ray crystallography, FRET analysis, and IR, Raman, CD, and in vitro NMR spectroscopy), the presented in-cell NMR approach only provides information on the behavior of short NA fragments. In other words, the in-cell NMR data do not reflect the influence of chromatin context and packing. However, the fact that DNA-binding ligands generally do not act on the closed chromatin but rather during processes involving its open state such as transcription and/or replication provides justification for the use of cells transfected with DNA-ligand complex as a model recapitulating the effect of DNA-targeting ligands in vivo.

Finally, the setup of the presented in-cell NMR approach for monitoring DNA-ligand interactions is conceptually distinct from those typically employed in in-cell NMR studies of interactions between ligands and protein-based targets. The approaches for in-cell NMR investigations of ligand-protein interactions use a combination of separate invasive delivery (or the overexpression) of the protein target into/in cells followed by noninvasive delivery of the ligand into the intracellular space (see recent review by Kang). In this case, the in-cell NMR readout not only reports on the impact of the intracellular environment on interaction between a protein and ligand, but it also reflects the uptake efficiency of the ligand by the cells. Therefore, in contrast to the invasive approach presented here, the hybrid approaches cannot be directly employed for comparison of several ligands binding to a single protein target in cells, since the distinct ligands generally have different uptake efficiencies.

**Conclusions**

The characterization of DNA-ligand complexes using in-cell NMR spectroscopy provides a unique opportunity to elucidate the effects of the complex intracellular environment on ligand-DNA binding. This technique can thus provide an important complement to conventional in vitro analyses, which cannot reflect the influence of factors present in intracellular space. The information provided by in-cell NMR spectroscopy is qualitatively distinct from that commonly derived under in vitro conditions. Commonly used descriptors of interactions such as dissociation constant and thermodynamic stabilization (corresponding to the difference between $T_m$ of the DNA target and that of the complex), while quantitative and extremely useful for describing the behavior of a two-component system comprising the ligand and its DNA target, are not reliable indicators of the behavior of the ligand-target system in the presence of dozens-to-hundreds of potential ligand competitors and environmental factors, which are generally poorly understood but are present in intracellular space of living cells. In the complex environment of a living cell, the capacity of a ligand to bind a DNA target is not only a function of the association and dissociation kinetics between the ligand and the DNA target or the thermodynamic stability of the DNA-ligand complex but also a function of all the kinetic processes and thermodynamic stabilities of all possible complexes involving the DNA or the ligand. The information obtained from in-cell NMR spectra can thus be taken as a “macroscopic/global phenotype” reflecting behavior of the DNA-ligand complex in the complex intracellular space.

The presented approach can be directly extended to RNA-based targets. The approach is also general and 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 and in-cell FRET techniques. In-cell NMR spectra of NA-ligand complexes might serve as an important (essential) benchmark for the formulation of simplified in vitro conditions/buffers for conventional in vitro spectroscopic methods or to assess the physiological relevance of in vitro data. One of the main potential applications of in-cell NMR spectroscopy of NAs in mammalian cells might be in complementing the established approaches employed in the drug development process. Although, the relative complexity of the method precludes
its full automation and relegates it to low-throughput applications, it has the potential to be invaluable in late-stage drug discovery for the elimination of false positive hits from (in vitro) high-throughput screens and the identification/validation of lead compounds. We believe that the presented method represents an important step forward in the characterization of NA-ligand interactions under native conditions, which will enhance our understanding of the impact of the intracellular environment on biomolecular interactions.

ASSOCIATED CONTENT
Supporting Information.

REFERENCES

The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

Experimental Section - Material and Methods, Supporting Figures S1-S10, Explanatory Notes S1-S2 (PDF)

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Supporting Information

Monitoring DNA-ligand interactions in living human cells using high-resolution NMR spectroscopy.

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Materials and methods

DNA oligonucleotides

The oligonucleotide 5′-d(CGTCGTAGTGC)-3′ and its complement 5′-d(GCACTTCGACG)-3′, corresponding to the forward and reverse (FW and RV) strands, respectively, forming the double-stranded T-T mismatch-containing DNA, herein referred to as TT-DNA; 5′-d(GCGAATTCCGTCTCCGGAATTGC)-3′, herein referred to as MH-DNA; and FAM 5′-terminally labeled 5′-d(GCGAATTCCGTCTCCGGAATTGC)-3′, and 5′-d(CGTCGTAGTGC)-3′ were purchased from Sigma-Aldrich (USA). The oligonucleotides were dissolved in H2O and subjected to standard n-butanol precipitation to remove contaminants from the solid-state synthesis following the protocol described by Viskova et al.1 Briefly, 35 mL of n-butanol (Sigma-Aldrich) was added to ~1 mL of an aqueous solution of the DNA oligonucleotide. The resulting mixture was vigorously shaken for 10 min and then centrifuged at 30,000 x g for 1 h. After centrifugation, the supernatant, consisting of the water-butanol fraction, was removed, and the pellet was air-dried. The dried pellet was resuspended in 1 mL of H2O, and the oligonucleotide concentration was determined from its UV absorbance measured on a NanoDrop 2000c (Thermo Fisher Scientific). The aqueous solutions of the DNA oligonucleotides were used to prepare stock solutions of 3 mM TT-DNA or MH-DNA in 140 mM sodium phosphate, pH = 7.2, 5 mM KCl and 10 mM MgCl2, referred to as electroporation (EC) buffer. The stock solutions were annealed by heating to 95 °C for 10 min and then cooled to room temperature. An analogous procedure (with milder conditions for DNA annealing: heating to 60 °C for 5 min and then cooling the solution to room temperature) was used to prepare 100 µM solutions of fluorescently labeled TT-DNA (FAM-TT-DNA) and MH-DNA (FAM-MH-DNA) in the EC buffer. The TT-DNA and MH-DNA solutions used for electroporation were prepared by mixing the TT- or MH-DNA solution with the corresponding solutions of covalently modified TT- or MH-DNA analogs bearing the fluorophore (FAM) at their 5′-termini (of the FW strand for the TT-DNA) at a 30:1 molar ratio (nonmodified DNA:FAM-modified DNA).

DNA-binding ligands

Netropsin hydrochloride (Figure 1A) was purchased from Sigma-Aldrich (USA, Cat. No. N9653). Macrocyclic ligands (Figure 1B) were prepared as hydrochloride salts according to previously published procedure.2

Preparation DNA-ligand complexes

Netropsin, 2,7-BisNP-NH, 2,7-BisNP-O, and 1,5-BisNP-O were dissolved in MilliQ H2O to prepare 5 mM stock solutions. The stock solutions were used to prepare 310 µM solutions of the ligands in EC buffer. The complexes of (FAM)-TT- or (FAM)-MH-DNA and the individual ligands were prepared by mixing (FAM)-TT- or (FAM)-MH-DNA and the ligands in equal molar ratios. The formation of the individual complexes was confirmed using 1D 1H NMR spectroscopy.

Preparation of in-cell NMR samples

HeLa cells (Sigma-Aldrich, USA) were cultured in DMEM (without phenol red) (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, GE Life Sciences) and penicillin-streptomycin solution (100 units penicillin and 0.10 mg streptomycin/mL) (Sigma-Aldrich, USA) under a 5% CO2 atmosphere at 37 °C. The different DNA or DNA-ligand complexes were integrated into the HeLa cells by electroporation using the BTX-ECM 830 system (Harvard Apparatus, USA). Prior to electroporation, cells in the late log phase were washed with prewarmed 1x Dulbecco’s Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, USA) and harvested using 0.05% trypsin and 0.02% EDTA (Sigma-Aldrich, USA) in 1xDPBS. Harvested cells were centrifuged at 1000 rpm for 5 min. Pelleted cells were resuspended in prewarmed 1xDPBS and counted in a Bürker counting chamber. Approximately 1.3 x 10⁸ cells were used.
to prepare each NMR sample (DNA or DNA-ligand complex). Cells for each transfection were centrifuged (1000 rpm for 5 min). Pelleted cells were resuspended in 3.2 mL of the EC buffer (140 mM NaPO$_3$, 5 mM KCl, 10 mM MgCl$_2$, pH = 7.2) containing 310 μM (FAM)-DNA/DNA-ligand complex. The cell suspension was divided into eight 4-mm electroporation cuvettes (Cell Projects, UK). All samples were incubated on ice for 5 min prior to electroporation. To achieve maximum transfection efficiency, the electroporation was conducted using two square-wave pulses (100 μs/1000 V; 30 ms/350 V) separated by a 5 s interval. After electroporation, the cells were incubated for 2 min at room temperature (RT), transferred into preheated (RT) phenol-red-free Leibovitz L15-/-/ medium (no FBS/no antibiotics) and centrifuged at 1000 rpm for 5 min to remove any remaining non-incorporated DNA from the cell surfaces. Cells were resuspended in fresh, pre-heated (RT) L15-/-/ medium. A small portion of the cell suspension (~ 6 x 10$^5$ cells) was used for flow cytometric (FCM) analysis and confocal microscopy analysis (see below) to evaluate the cell viability and the level of integration and localization of the exogenous DNA. The rest of the cell suspension was centrifuged at 1000 rpm for 5 min. Upon removal of the supernatant, the resulting cell slurry was resuspended in 0.6 mL of preheated (RT) Leibovitz L15-/-/ containing 10% D$_2$O and transferred into a Shigemi NMR tube (Shigemi Co., Tokyo, Japan). Prior to NMR spectroscopy, the cells in the NMR tube were manually centrifuged using a “hand centrifuge” (CortecNet, France) to form a fluffy pellet at the bottom of the NMR tube.

**Preparation of ex vivo NMR samples**

The cellular metabolic, cytosolic, and intact nuclei fractions employed for the ex vivo NMR characterization of the complexes between the TT-DNA and 2,7-BisNP-NH, 2,7-BisNP-O, and 1,5-BisNP-O were prepared as described in Viskova et al.$^1$

**Flow cytometry**

For FCM analysis, ~10$^5$ cells were resuspended in 200 μL of DPBS buffer (Sigma-Aldrich, USA) supplemented with 2μL (1 mg/mL) of propidium iodide (PI) (Exbio, Czech Republic) to stain the apoptotic and dead cells or cells with compromised membrane integrity. Then, 10$^4$ HeLa cells were analyzed with a BD FACSVerse flow cytometer using BD FACSuite software (BD Biosciences, San Jose, CA, USA). To detect the fluorescently (FAM) labeled DNA and to evaluate the transfection efficiency, the excitation wavelength was 488 nm, and the emission was detected at 527/32 nm. PI was excited at 488 nm, and the emission was detected at 700/54 nm.

**Confocal microscopy**

For confocal microscopy, ~ 5 x 10$^5$ cells were transferred to a 35-mm glass bottomed dish (ibidi GmbH, Germany) pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich, USA) supplemented with 2 mL of Leibovitz L15-/-/ medium containing 1 μg/mL Hoechst (Sigma-Aldrich, USA) to visualize the cell nuclei. All microscopy images were obtained using a Zeiss LSM 800 confocal microscope with a 63x/1.2 C-Apochromat objective. Images were taken in transmission mode with an excitation wavelength of 488 nm, and the fluorescently (FAM) labeled DNA was detected at emission wavelengths of 480-700 nm. For Hoechst, an excitation wavelength of 405 nm was used, and the emission was monitored at 400-480 nm.

**NMR spectroscopy**

NMR spectra were measured at 600 MHz using a Bruker Avance III HD spectrometer (Bruker, Corporation, Billerica, MA, USA) equipped with a quadruple-resonance cryogenic probe at 20 °C. 1D in vitro $^1$H NMR spectra were acquired in EC buffer or in buffer A (10 mM sodium phosphate, pH=7.9, 10 mM KCl, 300 mM sucrose, 0.5% NP-40, 1 mM Na$_3$VO$_4$, 20 mM NaF, 0.5 mM DTT, cOmplete EDTA-free Tablets (Roche, Switzerland)). The DNA concentrations of the in vitro and ex vivo samples were 200 μM and 50 μM, respectively. In vitro 1D $^1$H NMR spectra were acquired with 128 scans using a 1D $^1$H JR-echo (1-1
echo) pulse sequence$^3$ with zero excitation set to the resonance of water and the excitation maximum set to 13 ppm. *Ex vivo* and in-cell 1D $^1$H NMR spectra were acquired with 4x 128 scans using same pulse sequences. The *ex vivo* and in-cell NMR measurements were set up to conclude at 40 minutes post DNA mixing into cellular extract and cell transfection, respectively. The *in vitro* spectra were base-line corrected and processed with the exponential apodization function with the line-broadening parameter set to 6. The *ex vivo* and in-cell NMR spectra were processed with the exponential apodization function with the line-broadening parameter set to 14. The NMR spectra were processed using MNova v12.0.0 (Mestrelab Research, Spain). After the acquisition of the in-cell NMR spectrum, the 1D $^1$H NMR spectrum of the supernatant in each NMR tube was measured (using the same NMR parameters as were used to acquire the in-cell NMR spectrum) to assess the leakage of the transfected DNA from the cells. The cells in the NMR tube were subjected to FCM analysis to assess the cell mortality in the course of the NMR experiments.
**Figure S1:** Confocal microscopy images (LEFT), double-staining (PI/FAM) FCM analysis (MIDDLE), and distribution of the FAM signal intensity (RIGHT) in cultures of HeLa cells transfected (A) with “MOCK” (EC buffer) in the first and second electroporation step; (B) with TT-DNA and individual ligands (2,7-BisNP-NH, 2,7-BisNP-O, and 1,5-BisNP-O) in the first and second electroporation steps, respectively; (C) with the individual ligands (2,7-BisNP-NH, 2,7-BisNP-O, and 1,5-BisNP-O) and TT-DNA in the first and second electroporation steps, respectively; and (D) with the preformed 1:1 TT-DNA:ligand complexes (TT-DNA:2,7-BisNP-NH, TT-DNA:2,7-BisNP-O, and TT-DNA:1,5-BisNP-O) in a single electroporation step. A recovery period of 1 h was used between sequential electroporations. For the meanings of the colors (confocal images) and numbers (FCM analysis), see the legend to Figure 2.
Figure S2: A), B), C), and D) distribution of the FAM signal intensity in cultures of HeLa cells transfected with 1:1 complexes of MH-DNA:netrospin (cf. Figure 3A), TT-DNA:2,7-BisNP-NH (cf. Figure 3D), TT-DNA:2,7-BisNP-O (cf. Figure 5A), and TT-DNA:1,5-BisNP-O (cf. Figure 5D), respectively.

Figure S3: A) 1D $^1$H in-cell NMR spectrum of nontransfected HeLa cells recorded using a JR-echo (1-1 echo) pulse sequence$^3$. B) Comparison of the imino regions (gray box) of the 1D $^1$H in-cell NMR spectra of nontransfected HeLa cells (black) and HeLa cells transfected with the TT-DNA:2,7-BisNP-NH complex (red). The in-cell NMR spectrum of the complex was acquired with 512 scans. C) The imino regions of the 1D $^1$H in vitro NMR spectra of the TT-DNA:2,7-BisNP-NH complex at various concentrations (indicated) of the complex in EC buffer (black). The corresponding region of the in-cell NMR spectrum of HeLa cells transfected with the TT-DNA:2,7-BisNP-NH complex (red). All the spectra presented in C) were acquired with 128 scans.
Figure S4: Comparison of the imino regions of the in vitro (black) and in-cell (red) NMR spectra of MH-DNA:netropsin (A) and TT-DNA:2,7-BisNP-NH (B) complexes. The in vitro NMR spectra were acquired at a DNA-ligand complex concentration of 200 µM in the EC buffer at 20°C. For information on the in-cell NMR experiments, cf. Figure 3.

Figure S5: A) Double-staining (PI/FAM) FCM analysis (LEFT) and confocal microscopy images (RIGHT) of cells transfected with (FAM)-TT-DNA following treatment with the cell membrane-disrupting buffer (buffer A) at the indicated time points post transfection. B) Analogous data from the control experiment performed in the presence of growth medium (Leibovitz L15 -/). C) Quantification of the FAM fluorescence of cells treated with the cell membrane-disrupting buffer and of cells incubated in the presence of the media (control).

Figure S6: The imino region of the 1D $^1$H in-cell NMR spectrum of HeLa cells transfected with TT-DNA (red) and the preformed TT-DNA:1,5-BisNP-O 1:1 complex (black).
Commentary to the Figure S6:

The overall appearance of the in-cell NMR spectrum of TT-DNA is characterized by a low signal-to-noise ratio as well as by the presence of broad overlapping signals (Figure S6, A1). We assume that the appearance of the in-cell NMR spectrum of TT-DNA is a manifestation of the conformational heterogeneity of the TT-DNA, and in particular, a manifestation of pronounced intramolecular dynamics (base-pair openings) at and in close proximity to the T-T mismatch site in the intracellular space. “Normalization” of the in-cell NMR spectral signature upon a single point (stabilizing) mutation converting the T-T mismatch into a T-A Watson-Crick pair (Figure A1), which supports this explanation.

Figure A1: (A) Superposition of the imino regions of the in vitro (black) and in-cell (red) 1D $^1$H NMR spectra of TT-DNA. (B) The imino regions of the in-cell 1D $^1$H NMR spectra of TT-DNA (red) and its analog (black) bearing a single point mutation in the RV strand (5'-d(GCACTACGACG)-3’) that converts the T-T mismatch into a regular T-A Watson-Crick base pair.

Figure S7: PANEL 1: The imino region of the in vitro 1D $^1$H NMR spectrum of the TT-DNA:2,7-BisNP-NH (A), TT-DNA:2,7-BisNP-O (B), and TT-DNA:1,5-BisNP-O (C) 1:1 complexes acquired in the EC buffer prior (black) and following (red) “mock” electroporation comprising two square pulses (100 µs/1000 V; 30 ms/350 V) separated by a 5 s delay. PANEL 2: Analogous data acquired in the presence of the cellular metabolic fraction containing the ligands’ native off-targets.
Figure S8: (A) Representative regions of the 1D $^1$H NMR spectra of 2,7-BisNP-NH (LEFT), 2,7-BisNP-O (MIDDLE), and 1,5-BisNP-O (RIGHT) acquired in D$_2$O at 20°C. (B) Identical regions of the 1D $^1$H NMR spectra of the cellular metabolic fraction in the absence (black) and in the presence of 200 µM ligand (grey). (C) Expansions of selected regions of the 1D $^1$H NMR spectra of the cellular metabolic fraction in the absence (red) and in the presence of 50 µM (grey) and 100 µM (black) ligand, respectively. Black arrows indicate the changes in the metabolite signals’ positions and intensities as a function of increasing ligand concentration.

Figure S9: The in vitro 1D $^1$H NMR spectra of 2,7-BisNP-NH (A), 2,7-BisNP-O (B), and 1,5-BisNP-O (C) acquired in the absence (red) and in the presence (black) of selected cellular metabolites (ATP, cAMP, GMP, fumarate, succinate, and malate). Changes in the positions and/or line widths of the ligand-specific signals are indicative of the interactions between the ligand and the metabolite. The red (transparent) boxes highlight the positions of the ligand-specific signals. The spectra were acquired in D$_2$O at 20°C.
**Figure S10:** The *in vitro* 1D $^1$H NMR spectra of the TT-DNA:2,7-BisNP-NH, TT-DNA:2,7-BisNP-O, and TT-DNA:1,5-BisNP-O complexes acquired in the absence (A) and in the presence (B) of 150 µg of ctDNA (Invitrogen). C) The *in vitro* 1D $^1$H NMR spectrum of TT-DNA. The spectra were acquired in EC buffer at a TT-DNA/TT-DNA:ligand complex concentration of 50 µM. The blue and green vertical dashed lines indicate the characteristic signals from the ligand-bound and unbound forms of TT-DNA, respectively.

**Explanatory Note S1:**

Netropsin and naphthalenophane were chosen as the model drug systems because in addition to their medicinal importance,2,4 the NMR signals of their ligand-bound and unbound forms with the corresponding DNA target are distinct, making these systems particularly well suited for demonstrating the presented in-cell NMR approach.

**Explanatory Note S2:**

The imino hydrogen signals from intracellular (endogenous) DNA/RNA and their constituents (nucleosides) were not detected in the in-cell NMR spectra because most of the naturally occurring nucleic acids are either in macromolecules (e.g., genomic DNA), their concentration is too low (e.g., ncRNA), or their imino protons are not involved in hydrogen bonding and their signals are lost as a result of fast exchange with the bulk water (nucleosides and nucleotides) – cf. ref.5

**REFERENCES**
