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A Convenient Thiazole Orange Fluorescence Assay for the Evaluation of DNA Duplex Hybridization Stability

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Abstract

Objective—A simple and rapid method for measuring the hybridization stability of duplexes of DNAs and other oligomers in different environments is described. When added to an oligomer duplex, the thiazole orange (TO) dye intercalates and in this state is fluorescent. Therefore, when duplex dissociation occurs, the release of TO results in a detectable change in fluorescence intensity. This assay was developed primarily to screen antisense oligomer duplexes that are stable in serum and in the cytoplasm but unstable in the presence of their target messenger RNA (mRNA).

Methods—The two antisense oligomers of this investigation were both 25 mer phosphorothioate (PS) DNAs, one directed against the RI α mRNA and the other directed against the *mdr1* mRNA. The former duplex was first used in the solution studies, in most cases duplexed with a 16 mer phosphodiester (PO) complementary DNA (i.e., PS-DNA25/PO-cDNA16). Both duplexes were then tested in a series of cell studies using SK-BR-3 (RI α +), KB-G2 (*mdr1*++), and KB-31 (*mdr1*+/-) cells.

Results—Preliminary measurements in solution showed that maximum fluorescence was achieved when more than ten TO molecules were bound to each duplex. When a 25 mer PO-DNA or PO-RNA with the base sequence of the RI α mRNA was added, the dramatic change in fluorescence intensity that followed signified dissociation of the antisense DNA from the study duplex and reassociation with the target DNA. Kinetic measurements showed that this process was completed in about 3 min. Fluorescent measurements of SK-BR-3 (RI α +) cells incubated at 37°C with the anti-RI α study duplex over time showed a maximum at the point where the loss of fluorescence due to dissociation of the study duplex, probably by an antisense mechanism, began to dominate over the increasing fluorescence due to continuing cellular accumulation. A similar result was observed in the KB-G2 (*mdr1*+) cells incubated with the anti-*mdr1* study duplex.

Conclusions—When study duplexes shown to be stable in serum were incubated with their target cells, the assay successfully detected evidence of dissociation, most likely by an antisense mechanism. Thus, a TO fluorescence assay has been developed that is capable of detecting the dissociation of DNA duplexes.

Keywords

Thiazole orange; Fluorescence assay; DNA duplex; Antisense mechanism

Introduction

Optical antisense targeting with fluorescent-conjugated DNA oligomers has the potential of improving target tissue to normal tissue ratios over that achievable by nuclear antisense imaging. When administered as a linear duplex of two fluorophore-conjugated oligomers arranged in a manner that inhibits fluorescence as the duplex and designed to dissociate only in the presence of its target messenger RNA (mRNA), the fluorescence signal should in principle be inhibited everywhere except in the target cell. These laboratories have demonstrated proof of this concept in a xenograft mouse model using a Cy5.5-conjugated 25 mer DNA hybridized to a BHQ3-conjugated 18 mer complementary DNA [1]. By combining a fluorophore-conjugated antisense DNA with a quencher-conjugated shorter complementary cDNA, fluorescence was inhibited both in cell culture and in tumored animals except in the presence of the target mRNA. However, optimization of this approach will require that duplex candidates be screened to select for hybridization stability under all conditions except in the presence of the target mRNA. We have developed a simple assay that relies upon thiazole orange (TO; Scheme 1) and that may be used to rapidly achieve this screen. The use of dyes to measure duplex formation is a common feature of real-time polymerase chain reaction (PCR) [2–5].

The two aromatic rings of TO are connected by a carbon–carbon single bond with free rotation and can resonate only when the rings are coplanar. Because of free rotation, the dye shows negligible fluorescence in aqueous solution. However, by intercalating between the nitrogenous bases of DNA or other oligomers such as peptide nucleic acids [6, 7], rotation becomes restricted, thus closing an important nonradiative decay channel [8]. The fluorescence quantum yield of TO has been reported to increase 18,900 times upon binding to DNA [9, 10]. Thus, this property provides a convenient means for screening candidate oligomer duplexes for stability.

We now report that the assay easily detected dissociation of duplexes consisting of a 25 mer antisense DNA and a 16 mer complementary DNA when incubated in cells expressing the target mRNA by what is most likely an antisense mechanism.

Materials and Methods

The TO was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as received. All phosphorothioate (PS) and phosphodiester (PO) DNAs were obtained from Integrated DNA Technologies (Coralville, IA, USA) as was the unmodified RNA, in this case RNase free and high-performance-liquid-chromatography-purified.

For the solution studies, the target sequence was a 25 mer region of the R1α mRNA and was obtained as both the 25 mer PO-DNA and the 25 mer RNA (all T should be replaced by U in the sequence): 5'-GAA GGC TGC CAG TGA GGA GGC ACG C (target-cDNA25, target-

RNA25). The study PS-DNA25 in the solution studies was directed against the RI α mRNA: 5'-GCG TGC CTC CTC ACT GGC AGC CTT C [11]. A random 25 mer PO-DNA was used as a control in the solution studies: 5'-GGG ATC GTT CAG AGT CTA CTA AAT A (random-DNA25).

For the cell studies, the above study PS-DNA25 was again used as a PS-DNA25/PO-cDNA16 duplex: 5'-GCG TGC CTC CTC ACT GGC AGC CTT C/5'-CAG TGA GGA GGC ACG C, along with a second study PS-DNA25 directed against the *mdr1* mRNA also as a PS-DNA25/PO-cDNA16 duplex: 5'-AAG ATC CAT CCC GAC CTC GCG CTC C/5'-GGT CGG GAT GGA TCT T. In addition, three control PS-DNA25/PO-cDNA16 duplexes were also prepared for use in the cell studies:

5'-TAT TTA GTAGAC TCT GAA CGA TCC C/5'-GGG ATC GTT CAG AGT C,
control 1

5'-GTC CGG ACG CTT CTC C GA CCT CTC G/5'-G GAG AAG CGT CCG GAC,
control 2

5'-ATC AAC CAC CGC ACC C GT CCT TGC G/5'-G GGT GCG GTG GTT GAT,
control 3

The control 2 and control 3 sequences were selected to contain the same number of A, T, C, and G bases as that of the target RI α mRNA and the target *mdr1* mRNA, respectively. The SK-BR-3 (RI α ++) cells were purchased from ATCC (Manassas, VA, USA). KB-G2 (*mdr1*+) and its parent cell line KB-31 (*mdr1*+/-) were a gift from Isamu Sugawara (Research Institute of Tuberculosis, Tokyo, Japan.). The SK-BR-3 cells were cultured and maintained in the McCoy's 5a medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. The KB-G2 and KB-31 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS. The cells were incubated with duplexes in 1% FBS medium.

All duplexes were prepared by mixing the two single strands at a 1:1 molar ratio in 150 mM phosphate buffer and heating at 95°C for 5 min to dissociate any intrastrand duplexes. A stock solution of TO at 200 μ M in 150 mM PBS was also prepared. Following addition of the TO, the samples could be measured immediately since equilibrium between TO and DNA duplexes occurs extremely rapidly even at room temperature [12, 13]. All fluorescent measurements were at room temperature.

The fluorescence intensity of each plate was measured on a fluorescence microplate reader (Safire, Tecan, Switzerland) using a 5 nm slit width except for the kinetic studies in which a Multilabel Counter (Victor V, PerkinElmer, Waltham) equipped with an injector and shaker was used. In all cases, 504 nm was used for excitation and 527 nm for emission. Measurements were made immediately after shaking.

Influence of TO Concentration

Fluorescence measurements were performed to evaluate the influence of TO concentration on duplex fluorescence. The study PS-DNA25 was hybridized with PO-cDNA16, PO-cDNA18, and the target-cDNA25 and each duplex was added from a 100 μ M stock solution

in 150 mM PBS to wells of a 96 well plate along with a 200 μ M solution of TO in 150 mM PBS to a final TO concentration between 0 and 20 μ M. The TO to DNA molar ratios ranged from 0 to 40. The final concentration of each duplex was 0.5 μ M. The study PS-DNA25 was also mixed with the random PO-DNA25 in the identical fashion. Each measurement was in triplicate.

Evidence of Duplex Dissociation

The ability of the TO assay to measure duplex dissociation in the presence of a target-cDNA25 was investigated. The duplex PS-DNA25/PO-cDNA16 was prepared in wells at 0.5 μ M in PBS buffer as before and mixed with 9 μ M TO. The fluorescence intensity was then measured immediately and at 10 min. At that point, the target-cDNA25 at 0.5 μ M in PBS was added in a volume that provided the same molar concentration to each well and the fluorescence observed every 10 min thereafter. The PBS and the random-DNA25 were added to additional wells in the identical fashion as controls. Since TO has been reported to intercalate in DNA duplexes at about one TO for every two base pairs [9], saturation may be expected at about 4 μ M concentration of TO. The 9 μ M was selected to provide an excess TO free in solution after addition to the duplexes of this investigation. In this way, an increase in fluorescence may be expected following dissociation of a 16 mer duplex and formation of a 25 mer duplex.

Comparison of DNA and RNA as Targets

The application of the TO assay of particular interest to this laboratory will be to screen various duplexes for instability in the presence of a target mRNA. However, the difficulty with native phosphodiester RNAs is their chemical instability to the ubiquitous presence of nucleases. To ensure that the more chemically stable DNA could be used for convenience in place of RNA as target for the screening, a direct comparison was performed. The native RNA had the same base sequence and chain length as the target-cDNA25 and the study was performed identically to that described above using the PS-DNA25/PO-cDNA16 duplex.

Kinetics of Duplex Dissociation

The Multilabel Counter can measure fluorescence over time intervals as short as 1 s and was used to measure the change of fluorescence signal every 6 s after injection of the target-cDNA25 to wells containing the PS-DNA25/PO-cDNA16 duplex. Identical wells were injected with the random-DNA25 and with an equal volume of PBS as controls.

Duplex Stability in Serum

The TO assay was then used to evaluate the stability of the study PS-DNA25/PO-cDNA16 duplex after incubation at 37°C in 70% normal mouse serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After 2 h of incubation, the TO was added to a final concentration of 9 μ M and the fluorescence intensity was measured at room temperature. As controls, the duplex was also incubated at room temperature in 70% serum and in PBS at 37°C.

Cell Studies—The TO assay was developed primarily as a simple method of measuring the instability in mRNA target cells of duplexes consisting of a longer antisense DNA hybridized to a smaller complementary DNA. A convincing demonstration of this property with the proper controls would show that the duplex was able to cross the cell membrane, was stable in the cytoplasm, and was unstable in the presence of its target mRNA. To minimize the possibility that a significant difference in any cellular property between an antisense and a control oligomer may confuse an aptameric mechanism for an antisense mechanism [14], two cell types were used along with two mRNA targets. The cells were SK-BR-3 and KB-G2 expressing the *RI α* and *mdr1* mRNAs, respectively. The SK-BR-3 cells were used with the study PS-DNA25 against the *RI α* mRNA duplexed with the PO-cDNA16 duplex and with control 1 and control 2 duplexes as controls whereas the KB-G2 cells were used with the study PS-DNA25 against the *mdr1* mRNA duplexed with PO-cDNA16 and with control 1 and control 3 duplexes as controls. KB-31 has been reported [15] to express the *mdr1* mRNA at low levels and was used as a negative control cell in this study. All three cell types were seeded in 96 well clear bottom microplates (Corning, Wilkes-Barre, PA) at 50,000 cells per well, incubated with 10% FBS culture medium overnight, and then incubated in triplicate for up to 3 h at 37°C with the study or control duplexes at 0.5 μ M after the earlier addition of TO at 3 μ M in 1% FBS. The 3 μ M TO concentration used in the cell studies was lower than the 9 μ M concentration used in the solution studies because preliminary measurements in cells showed lower background fluorescence at the lower TO dosage. As a result of the lower TO concentration, no excess TO was present during the cell incubations. The cells were then washed twice with 1% FBS culture medium and incubated for 1 h in 1% FBS culture medium before being washed twice with PBS. The cells were then lysed by the lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) at 100 μ L per well before the fluorescence was measured on the fluorescence microplate reader at room temperature.

Results

Influence of TO Concentration

As shown in Fig. 1, the fluorescence of all three study duplexes (traces a to c) increased steeply with increasing TO concentrations initially and reached a maximum at about 9 μ M for the longer PS-DNA25/target-cDNA25 duplex and at about 4 μ M for both shorter duplexes. This difference in maximum TO concentration was expected since TO has been reported to intercalate in duplexes at about one molecule for every two base pairs [9]. With increasing TO concentration, the fluorescence intensity then decreased steadily in each case probably due to self-quenching by electrostatically bound TO as the number of TO molecules per duplex increased and the distance between TO molecules decreased. As expected, the fluorescence was lowest in the PS-DNA25/random-DNA25 solution (trace d), probably due to weak binding affinity between the two noncomplementary DNAs. In the absence of any duplex, the fluorescence intensity of the free TO was essentially zero (trace e) over the concentration range examined. These results show that in the subsequent solution studies using duplexes shorter than the PS-DNA25/target-cDNA25, the 9 μ M TO concentration would therefore be in excess and would provide extra TO for intercalation with additional base pairs.

Evidence of Duplex Dissociation

The ability of the assay to measure instability of the study duplex towards a target cDNA in solution was examined. Figure 2 shows the change of fluorescence intensity in a solution of the PS-DNA25/PO-cDNA16 duplex following the injection of the target-cDNA25 (trace a). The dramatic increase in fluorescence intensity following the addition is a result of the dissociation of the PS-DNA25/PO-cDNA16 duplex and the formation of the new PS-DNA25/target-cDNA25 duplex. The fluorescence increases because the longer 25 mer duplex that is now present in solution provides additional binding sites for both the released and excess TO. Adding the random-DNA25 in place of the target-cDNA25 also shows some increase of fluorescence probably due to accidental but limited base pairing (trace b). The fluorescent response was essentially unchanged with the injection of PBS buffer at the same volume (trace c). These results demonstrate that the assay is capable of detecting duplex dissociation.

Comparison of DNA and RNA as Targets

A comparison was performed to ensure that DNA can be used in place of RNA for convenience as target for the screening of duplexes intended for mRNA binding. Figure 3 presents the measured fluorescence intensity as a function of reaction time following the addition at 20 min of either the target-cDNA25 or target-RNA25 with the same sequence to the wells containing the duplex PS-DNA25/PO-cDNA16. There are no significant differences at any time point (Student's *t* test, $p>0.05$).

Kinetics of Duplex Dissociation

Once again, the PS-DNA25/PO-cDNA16 duplex at 0.5 μ M in PBS was mixed in wells with 9 μ M TO followed by the target-cDNA25 added to the same 0.5 μ M concentration. The fluorescence intensity was measured every 6 s. Since the equilibrium between TO and DNA duplexes is extremely rapid [12, 13], this measurement can provide an accurate estimate of the kinetics of duplex dissociation. As shown in Fig. 4, the dissociation of the duplex PS-DNA25/PO-cDNA16 and the formation of the new PS-DNA25/target-cDNA25 duplex was completed in about 3 min. The injection of a random-DNA25 or buffer did not have any appreciable effect on the response.

Duplex Stability in Serum

The PS-DNA25/PO-cDNA16 duplex at 0.5 μ M was incubated at 37°C in 70% normal mouse serum for 2 h before addition of TO to a final concentration of 9 μ M at a ratio of 18 TO molecules to one duplex and the fluorescence intensities were measured at room temperature. There were no significant differences among the intensities suggesting that the duplex is stable over 2 h at 37°C in serum (data not presented). The results indicate that the TO fluorescence assay can be used to monitor the stability of DNA duplexes in 37°C serum, as an approximation of *in vivo* stability.

Cell Studies

Unlike the fluorescent studies in solution, the fluorescence in cells cannot increase with duplex dissociation since by using a 3 μ M rather than a 9 μ M TO concentration, there was

no free TO in the incubation. Therefore, the excess TO that is responsible for the fluorescence increase in the solution studies will not be present in the cell studies. Figure 5a shows the SK-BR-3 cellular fluorescence intensity following incubation with the study anti-RI α mRNA antisense PS-DNA25/PO-cDNA16 duplex and controls. The fluorescence intensity of cells incubated with both controls progressively and linearly increased with incubation time, due to continuing cellular accumulation of the fluorescent duplex. The fluorescence intensity of the cells incubated with the study duplex also increased initially, again due to increasing cellular accumulation, but reached a maximum after about 60 min. Presumably, the cellular accumulations of the study duplex continued to accumulate beyond this time as in the case of both control duplexes; nevertheless, the fluorescence intensity decreased thereafter. The most likely interpretation is that, after about 60 min, the loss of fluorescence due to dissociation of the study duplex by an antisense mechanism began to dominate over the increasing fluorescence due to continuing accumulation. The lower fluorescence in cells incubated with the study duplex became significant compared to cells incubated with either control at 120 min and thereafter.

As shown in Fig. 5b, the same trends, although not quite as dramatic, occurred in KB-G2 cells in that the fluorescence intensity increased linearly with time in cells incubated with both control duplexes and also initially for the study duplex as well. However, the increasing fluorescence of cells incubated with the study duplex slowed and again became significant at 120 min and thereafter.

To provide further evidence that fluorescence changes in cells incubated with a study duplex may be due to dissociation by an antisense mechanism, the fluorescence of the low-level *mdr1* expressing KB-31 cells were compared to that of the high-level KB-G2 cells. After adding TO, the anti-*mdr1* duplex or control 1 or control 3 duplexes were incubated with both cell types for 3 h at 37°C. As shown in Fig. 6, no significant differences in fluorescence were observed in KB-31 cells regardless of duplex. Furthermore, no significant differences in fluorescence of KB-G2 cells were observed between control duplexes. However, the fluorescence of KB-G2 cells incubated with the study duplex was significant lower ($p<0.05$) compared to KB-G2 cells incubated with either control duplex. That the fluorescence in KB-G2 cells compared to KB-31 cells was lower regardless of duplex is most likely due to differences in accumulations between cell types.

Discussion

The need for a simple and rapid method of evaluating duplex stability in different environments led to the development of the assay described herein. Although surface plasmon resonance is also capable of measuring duplex stability, this technique requires immobilization of the target strand and cannot be used in serum environments. By comparison, the TO assay does not require conjugation and should be useful in most biological environments. The property of TO that will explain its selection for the assay is its ability to intercalate between the strands of homogenous DNA oligomers as well as heterogeneous oligomers such as DNA/PNA [6, 7]. Thus, the fluorescence for a study duplex bound with TO will confirm that the duplex is intact and, conversely, a change in fluorescence intensity will confirm that duplex dissociation has occurred. The 527 nm

fluorescence of TO was convenient for this study but is far removed from the near-infrared frequencies; thus, the assay will be of limited use *in vivo*. However, dyes other than TO have similar properties and more favorable fluorescence frequencies [16–18].

One concern in the use of the TO assay to measure hybridization stability is whether the presence of multiple TO molecules in the duplex itself will influence stability. Previous reports have shown that the intercalation of fluorescence dyes such as SYBR Green I [3, 19], EB [20, 21], BEBO [22], YO-PRO-1 [23], LC Green [24], and SYTO-9 [5, 25] have often been used in real-time PCR and melting temperature analysis even though these dyes all intercalate into DNA between base pairs similar to that of TO. Recently, the TO analogs SYTO-82, SYTO-64, and SYTO-13 have been shown to have no influence on duplex melting temperature even at high concentrations (20 μ M) [26]. Two other relatives, TO-PRO-3 and YO-PRO-1, showed a melting temperature increase of roughly 5°C following intercalation but only at concentrations of 20 μ M. Since a concentration no higher than 9 μ M was used in this research, it is likely that the TO had only minimal if any influence on duplex stability.

In the solutions studies, an excess of TO was added to each duplex to provide an increasing fluorescence signal following binding of both the excess TO and the released TO to the newly formed and longer duplex. Since an excess of TO was not used in the cell studies, it was possible that no change in fluorescence signal would be observed following duplex dissociation. However, as shown in Figs. 5 and 6, the fluorescence intensity actually decreased following dissociation. It is likely that the TO, when released in the cytoplasm of cells, unlike when released in solution, is cleared or otherwise inhibited from binding to newly formed (or endogenous) duplexes. Thus, the increasing fluorescence intensity resulting from the continuing accumulation of the fluorescent duplexes in cells combines with a decreasing fluorescence due to duplex dissociation to result either in an overall decrease in signal over time as in Fig. 5a or an unchanged signal as in Fig. 5b.

Thus far, the assay has only been used with DNA duplexes. However, since the TO intercalates between base pairs and since both DNA and synthetic oligomers such as phosphorodiamidate morpholinos, locked nucleic acids, etc. all position the bases identically in their structure, the assay may be largely independent of oligomer backbone. That the target DNA and RNA provided identical results (Fig. 3) supports this suggestion.

Conclusions

In conclusion, a duplex stability assay has been developed that is capable of detecting duplex instability leading to dissociation both in solution and, more importantly, in cells. The assay will be applied in this laboratory to screen oligomer duplexes for optical antisense imaging applications.

Acknowledgments

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Fig. 1. Fluorescence measurements of different duplexes (a) PS-DNA25/target-cDNA25, (b) PS-DNA25/PO-cDNA18, (c) PS-DNA25/PO-cDNA16, (d) PS-DNA25/random-DNA25, all at 0.5 μM , and (e) 150 mM PBS buffer, as a function of the TO concentration. *Error bars* represent standard deviations for three replicates.

Fig. 2.

Fluorescence measurements showing the dissociation of the PS-DNA25/PO-cDNA16 study duplex and the formation of (Arrow) the PS-DNA25/target-cDNA25 duplex after injection of target-cDNA25 (*trace a*) at 10 min. The injections of random-DNA25 (*trace b*) and PBS buffer (*trace c*) were used as control. *Error bars* represent standard deviations for three replicates.

Fig. 3. Fluorescence measurements showing the dissociation of the PS-DNA25/PO-cDNA16 duplex and the formation of the new duplex (PS-DNA25/target-cDNA25 or PS-DNA25/target-RNA) after injection of target-cDNA25 or target-RNA25 with the same base sequence. *Error bars* represent standard deviations for three replicates.

Fig. 4. Kinetics of PS-DNA25/PO-cDNA16 duplex dissociation in the presence of target-cDNA25, random-DNA25, or buffer.

Fig. 5. Fluorescence intensities in SK-BR-3 (a) and KB-G2 (b) cells after incubation with study compared to control duplexes for different times. *Error bars* represent standard deviations for three replicates.

Fig. 6. Fluorescence intensity in KB-G2 (mdr1++) and KB-31 (mdr1+/-) cells after 3 h of incubation with 0.5 μ M of the anti-mdr1 study or control duplexes. *Error bars* represent standard deviations for three replicates.

Scheme 1.
Chemical structure of thiazole orange.