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Morphological Effects of G-Quadruplex Stabilization Using a Small Molecule in Zebrafish

Tani Agarwal,[†] Mukesh Kumar Lalwani,[†] Santosh Kumar,[†] Saumya Roy,[‡] Tushar Kanti Chakraborty,*[,‡,§] Sridhar Sivasubbu,*[,†] and Souvik Maiti*[,†,∥]

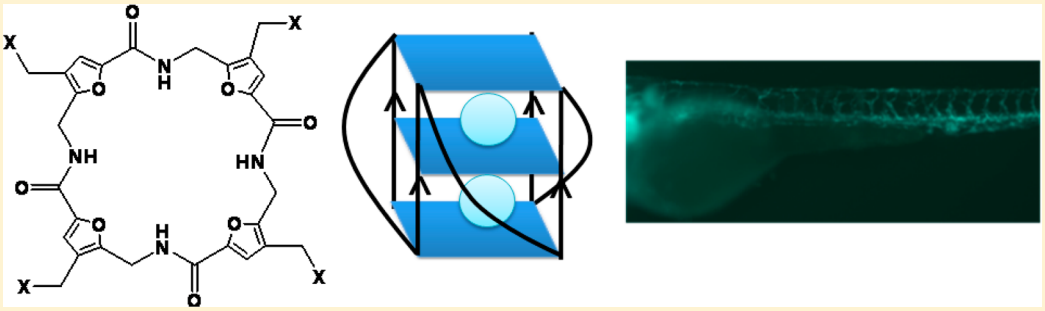
[†]CSIR-Institute of Genomics and Integrative Biology, , Mall Road, New Delhi 110 007, India

[‡]CSIR-Indian Institute of Chemical Technology, , Hyderabad 500 007, India

[§]Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India

[∥]CSIR-National Chemical Laboratory, Pune 411008, India

Supporting Information



ABSTRACT: Zebrafish (*Danio rerio*) embryos are transparent and advantageous for studying early developmental changes due to *ex utero* development, making them an appropriate model for studying gene expression changes as a result of molecular targeting. Zebrafish embryos were injected with a previously reported G-quadruplex selective ligand, and the phenotypic changes were recorded. We report marked discrepancies in the development of intersegmental vessels. *In silico* analysis determined that the putative G-quadruplex motif occur in the upstream promoter region of the Cdh5 (N-cadherin) gene. A real-time polymerase chain reaction-based investigation indicated that in zebrafish, CDH-2 (ZN-cad) was significantly downregulated in the ligand-treated embryos. Biophysical characterization of the interaction of the ligand with the G-quadruplex motif found in this promoter yielded strong binding and stabilization of the G-quadruplex with this ligand. Hence, we report for the first time the phenotypic impact of G-quadruplex targeting with a ligand in a vertebrate organism. This study has unveiled not only G-quadruplex targeting in non-human animal species but also the potential that G-quadruplexes can provide a ready tool for understanding the phenotypic effects of targeting certain important genes involved in differentiation and developmental processes in a living eukaryotic organism.

Several G-quadruplex selective small molecule ligands[1−9] have been used independently to stabilize cellular G-quadruplexes and mediate downregulation of several genes such as c-MYC, c-KIT, VEGF, PDGF, BCL-2, HIF, etc. Although G-quadruplex-mediated gene regulation is well-documented in human proto-oncogenes, it would be of interest to go a step further and to also look at other functional manifestations of G-quadruplex-altered gene expression. Apart from the human genome, these structures have been reported in other organisms such as *Oxytricha* sp., *Stylonychia lemnae*, *Saccharomyces cerevisiae*, and prokaryotes such as *Escherichia coli*, *Streptomyces*, etc.[10,11] For decades, G-quadruplex research has been focused on prokaryotes, yeast, or cell culture models. In fact, there has been no report of the study of the role of the G-quadruplex in higher eukaryotic organisms. This study is a first step in examining G-quadruplex-based gene regulation in a eukaryotic system. Hence, it will be of particular interest to observe closely the direct effect of G-quadruplex stabilization in a eukaryotic model organism, zebrafish.

The zebrafish as a model organism provides a valuable system for studying various molecular mechanisms and understanding biological pathways.[12,13] The main reasons are the small size, experimental accessibility, and ease in handling the animals. Also significant is the production of a large number of embryos, their optical clarity, and their rapid development. Because of these physical advantages, the zebrafish has quickly become a model of choice, especially for understanding embryonic development in vertebrate biology.[12] Additionally, we suggest zebrafish may serve as a valuable tool in studying the biological effects of G-quadruplex ligands as it provides a rapid

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platform for studying the molecular effects uisng optically clear embryos as the phenotypic manifestations are easily visible within a few hours of fertilization in the early developmental processes of zebrafish. In this study, we particularly sought to study the cardiovascular system, as it is one of the first organ systems to appear and take shape during embryonic development and retains morphogenetic plasticity during adult life.[14−16] Furthermore, vasculature can be easily visualized in live and optically clear zebrafish embryos using endothelial specific expression of fluorescent proteins.[17−19] Thus, it becomes feasible to follow early events in cardiovascular development and also to closely study wild-type and mutant phenotypes for several days, more so because oxygenation in the early zebrafish embryo is independent of blood circulation.[20] Most importantly, functional studies have shown that the molecular components and mechanisms that regulate vascular development are well conserved between mammals and fish.[21−23] Thus, the zebrafish embryo allows us to understand the developmental intricacies, and live imaging combined with functional studies makes it possible to gain a holistic understanding of the molecular mechanisms responsible for morphogenetic expression.

N-Cadherin plays important roles in neural and cardiovascular development, including somitogenesis, heart morphogenesis, neural tube formation, and early establishment of left−right asymmetry in vertebrate organisms.[24−26] Cadherins allow cell−cell adhesion required during embryonic development,[27] and N-cadherin (N-cad) is often one of the first expressed cell adhesion proteins during this process.[28−31] Recent studies demonstrate that N-cadherin or cadherin 2 is involved in the interaction of endothelial cells with mural cells like the pericytes and vascular myocytes, thereby recruiting them to the morphogenesis of the developing vasculature.[32,33] This leads to the proper maturation and stabilization of the emergent vasculature. Concomitant with the integral role of N-cadherin in the development of vasculature and angiogenesis are the observations that insufficient vascular growth may also contribute to neurodegeneration, suggesting a central role of N-cadherin in neural development also.[31,34]

We describe a novel G-quadruplex found in the N-cadherin gene in zebrafish (ZN-cad) and its interaction with a previously reported furan-based cyclic homo-oligopeptide ligand.[9] We have also transcended G-quadruplex-mediated gene regulation to a novel realm of its role in developmentally important genes and most importantly the direct visualization of its morphogenetic effect. Strikingly, a nontelomeric G-quadruplex is described in a non-cancer model. Most importantly, the unique implication in this study is the first demonstration of the morphological effect of G-quadruplex stabilization in a live animal model.

MATERIALS AND METHODS

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded in a Jasco spectropolarimeter (model J-715) equipped with a thermoelectrically controlled cell holder and a cuvette with a path length of 1 cm. The oligonucleotides were heated at 95 °C for 5 min followed by slow programmed cooling (0.2 °C/min) in 10 mM Tris buffer (pH 7.4) without KCl or with 25 or 100 mM KCl (as described for individual experiments). CD spectra for G-quadruplexes (5 μM) were recorded between 220 and 320 nm at 25 °C, and the average spectra of three scans are reported.

CD Melting Studies. The ZN-cad quadruplex sample was prepared by heating the oligonucleotides in 10 mM sodium cacodylate buffer (pH 7.4) and 25 mM KCl followed by slow cooling. The melting experiment was performed at a concentration of 5 μM in a Jasco spectropolarimeter (model 715) equipped with a thermoelectrically controlled cell holder and a cuvette with a path length of 1 cm in the absence and presence of different molar ratios of ligands at a heating rate of 0.2 °C/min. Data were collected at 264 nm and normalized to the CD signal obtained at 20 °C to obtain the reverse sigmoidal melting traces. Typically, three replicate experiments were performed, and average values are reported.

Isothermal Titration Calorimetry (ITC). ITC measurements were performed in a VP-ITC titration calorimeter (MicroCal, Northampton, MA). Before being loaded, the solutions were thoroughly degassed. The preformed quadruplex (10 μM) was kept in the sample cell, and the ligand (300 μM) in the same buffer was added to fill the syringe with a volume of 300 μL. The ligand solution was added sequentially in 10 μL aliquots (for a total of 25 injections, 20 s duration each) at 3 min intervals at 25 °C. Sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell until the titration signal was essentially constant. The heats of dilution were determined in parallel experiments by injecting a ligand solution at the same concentration in the same buffer. The respective heats of dilution were subtracted from the values from the corresponding binding experiments prior to curve fitting. Typically, three replicate titration experiments were performed.

Ligand Injections in Embryos and Maintenance. Zebrafish used in this study were housed at the CSIR Institute of Genomics and Integrative Biology following standard husbandry practices.[46] Transgenic zebrafish embryos were obtained by pairwise mating of adults. Double-transgenic zebrafish [*Tg*(*fli1:EGFP, gata1:RFP*)] expressing red fluorescent protein in blood cells and green fluorescent protein in all types of ECs were used in this study. Embryos at the single-cell stage were harvested and injected with a 3 nL dose of different concentrations of ligand and N-cadherin morpholino. The sequence of morpholino, adapted from ZFIN (MO1-cdh2), was 5′-TCTGTATAAAGAAACCGATAGAGTT-3′, and the sequence of the control morpholino was 5′-CCTCTTACCT-CAGTTACAATTTATA-3′. The morpholinos were procured from Gene Tools and used according to the manufacturer's instructions. The injected embryos were reared in 1-phenyl-2-thiourea (PTU)-treated embryo water to prevent pigmentation, and the water was changed every 24 h; survival was also assessed at the same time.

At 72 h postfertilization, the final scoring and imaging were conducted using a Zeiss Axioscope 40 fluorescent microscope (Carl Zeiss) using a 2.5× magnification and a 0.075 numerical aperture. Animals were treated with phenylthiourea to inhibit pigment formation. Images were processed with Zeiss AxioVision version 4.6 and Adobe Photoshop CS. Identical modifications and adjustments were applied to all the images in the same experiment.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR). Total RNA was isolated from 30 embryos using Tri-Reagent (Sigma Chemical Co.) as recommended by the manufacturer. DNase treatment was performed according to the manufacturer's instructions using DNase from Invitrogen. Total RNA (1 μg) was reverse transcribed in a 20

μL reaction volume using Fermentas Revert Aid Reverse Transcriptase enzyme. The cDNA was hence amplified using SYBR Green Real-Time PCR Master Mix from Kapa in the real-time PCRs. Amplification was conducted in an LC 480 instrument from Roche with the following cycling conditions for PCR: 94 °C for 2 min, followed by 45 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Amplification of β-actin was performed as a control using the same PCR conditions. The primers were 5′-TGAGGAGACACCGCCCAC-3′ and 5′-CAACATCGATTTCTTCCTCATCTTC-3′ for ZN-cad and 5′-TGCTGTCTCCATGTTTGATGTATCT-3′ and 5′-TCTCTGCTCCCCACCTCTAAGT-3′ for β-actin.

Ethics Statement. Fish experiments were performed in strict accordance with the recommendations and guidelines published by the CSIR Institute of Genomics and Integrative Biology. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the CSIR Institute of Genomics and Integrative Biology (Proposal 45a). All efforts were made to minimize animal suffering.

RESULTS AND DISCUSSION

The past decade has seen the publication of a plethora of evidence highlighting the role of G-quadruplexes in the modulation of gene expression. While a majority of the studies have demonstrated DNA G-quadruplexes in human telomeric regions and proto-oncogene promoters, only few reports have investigated G-quadruplexes in other species like *Caenorhabditis elegans*, *S. cerevisiae*, and *E. coli*.[10,11] Strikingly, G-quadruplexes in non-human vertebrates have remained largely elusive. This study focuses on a novel G-quadruplex found in the promoter of the N-cadherin gene of zebrafish (*Danio rerio*) (ZN-cad). We highlight here the functional relevance of this G-quadruplex and also demonstrate the biological effects of targeting it with a previously reported highly selective G-quadruplex binding small molecule ligand. This study is a significant advance in the field as it is the first demonstration of the morphological effects of chemical targeting of a single G-quadruplex in a whole living animal. The study aptly focuses on the utilization and application of a biological animal model to investigate the fine chemical underpinnings behind the small molecule-derived stabilization of the G-quadruplex in gene regulation.

The G-Quadruplex Motif in the ZN-cad Promoter. The N-cadherin gene is located on chromosome 20 in the zebrafish genome. A bioinformatic search using Quadfinder[35] across a genomic region up to 2 kb upstream from the start site of the gene predicted a putative intramolecular G-quadruplex with a contiguous array of guanines. This novel 24-nucleotide G-quadruplex was found ∼225 bases upstream of the transcription start site (TSS) of the N-cadherin or CDH2 gene of the zebrafish (ZN-cad) (Figure 1). This region most probably corresponds to a proximal promoter upstream of the start site of the ZN-cad gene as also confirmed from server-based searches of eukaryotic promoters. Also, this sequence could possibly form a G3 quadruplex with three loops of six, four, and one or two bases, GGGCATTACGGGGGCAGGGTGGGG.

It is noteworthy here that the human orthologue of the same gene is devoid of this promoter quadruplex. Moreover, the other well-researched G-quadruplex motifs found in human promoters like those in c-MYC and VEGF are surprisingly not found in zebrafish genome. However, in light of studies reporting on the relatively GC content of the genome of zebrafish,[36] it is quite possible that only few promoter G-quadruplexes may be present. This makes for a favorable G-quadruplex specific model system in which additive and/or contrasting effects arising from too many individual G-quadruplexes in the genome can be avoided naturally.

AGCACAGCATCTGAATTTCAAAGCAGCACACCAGCATGAGACCTGCA
CTTTAAAATCCAGGAATTCTCAGTCCCTCTTGCCGATTAGTGGGCA
TTACGGGGGCAGGGTGGGGTTAAAGAAAGAAGATCTTCCTTTT
GTTGTAGTGAAGAAGGCGGAGTCTTGTCTTCTTCGCTCTGACTTCCG
GAGGCTGAAAGGGGTGACATAAGGAAAGGAGCCCCCTTCTTTGCCG
CAGTACCAATGGGTGCGGCTAGAGGGCGTCATTGAGAGCGTGGCGC
TGCCGTTGAACAGCGAGAGCGAGCGGAGCTTCAGTACCAGACCGAG
CGCAGCACGGATCAGTGCCAGAGAGAGACGGAGGAACGATTCTGTC
GCTTCATGCCGCCGCTACCGTACGGAACATTATAACCTAGCGGAAAA
CGGCCAGCTCTATCGGTTTCTTTATACAGAACGGAATTTAAACGATG
TACCGTTCCGGAGGCGTGATGCTGGGGCTTCTCGCCGCTCTGCAG

Figure 1. Zebrafish N-cadherin proximal promoter sequence extracted from Ensembl. This figure shows the 24-nucleotide sequence of the G-quadruplex motif highlighted in a larger font, and the guanines in quartets are underlined. The red sequence is the 5′ UTR of the zebrafish N-cadherin gene. The subsequent sequence shows the coding sequence in black.

Characterizing the ZN-cad Proximal Promoter G-Quadruplex Motif. The guanine-rich sequence identified in zebrafish N-cadherin (ZN-cad) was investigated for G-quadruplex formation by biophysical methods in a 24-nucleotide oligonucleotide sequence. CD spectroscopy, which is a fundamental technique for characterization of secondary structures of biomolecules *in vitro*, was performed with the 24mer oligonucleotide sequence separately in the presence of increasing concentrations of K^+ and Na^+ ions in the buffer (Figure 2a,b). It was observed that in the presence of K^+ ions, an altogether different CD spectrum was obtained with a sharp positive peak at 262 nm and a negative peak at 240 nm (Figure 2a) characteristic of the parallel topology of the G-quadruplex,

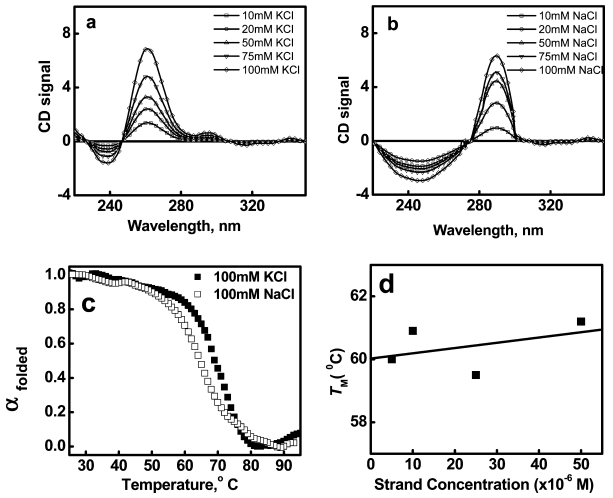

Figure 2. Characterization of the zebrafish N-cadherin G-quadruplex. Panels a and b show CD spectra of 24mer G-quadruplex forming sequence in the presence of 10, 20, 50, 75, and 100 mM K^+ and Na^+ ions separately showing differential folding behavior. (c) The UV melting experiment in 100 mM Na^+ and K^+ separately demonstrated differential thermal stability under different salt conditions. (d) The T_m obtained from the UV melting experiment performed at different strand concentrations remained constant, showing the formation of the unimolecular G-quadruplex at 25 mM KCl. All experiments were performed in 10 mM sodium cacodylate (pH 7.0). Each experiment was repeated thrice, and average values within a 5−10% error range are reported.

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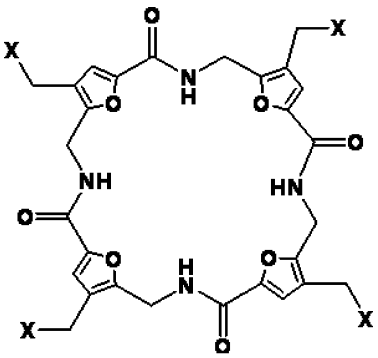
whereas in the presence of Na^+, the CD spectrum displayed a positive peak at 293 nm and a small negative signal at 240 nm (Figure 2b) indicative of the antiparallel G-quadruplex signature. Such disparity in folding topology with different monovalent cations suggested salt dependence. G-Quadruplexes are typical secondary structures whose folding, topology, and stability are governed by surrounding salt conditions.[37] Hence, it is plausible that the folded secondary structure formed by the G-rich sequence demonstrates G-quadruplex behavior.

Additionally, in a UV melting assay, the existence of the G-quadruplex was also evident from the hypochromicity at 295 nm observed with an increasing temperature in the melting traces of the G-rich oligonucleotide (Figure 2b). Further, the salt-dependent behavior was also verified by performing the UV melting experiment in the presence of either 100 mM Na^+ or K^+ ions. The melting traces depicted in Figure 2c also demonstrated the differential stability of the G-quadruplex under different ion conditions. In a strand concentration-dependent melting experiment, the T_m remained almost constant for strand concentrations of 5, 10, 25, and 50 μM, at around 60 ± 1 °C in the presence of 25 mM KCl to near the intracellular K^+ concentration in zebrafish.[38] This experiment demonstrated the formation of a stable intramolecular G-quadruplex. Overall, it was confirmed by a biophysical study that the secondary structure assumed by the G-rich 24mer oligonucleotide in this study was a stable intramolecular G-quadruplex with a parallel topology in the presence of K^+ ions. Moreover, in a Native PAGE experiment, it was seen that the G-quadruplex molecules migrate as a single population, indicating the presence of a single predominant conformer (Figure 1 of the Supporting Information). The 24mer intramolecular G-quadruplex formed in the presence of K^+ ions migrates ahead of the size marker because of the compactness of the G-quadruplex.

Interaction of the ZN-cad G-Quadruplex with a Selective Ligand. A previously reported furan-based cyclic homo-oligopeptide (Scheme 1) with demonstrated selectivity for the G-quadruplex was chosen to assess the binding and stabilization of the ZN-cad G-quadruplex. The same ligand had been demonstrated in previous studies to selectively stabilize the G-quadruplex and repress downstream transcription in the c-MYC gene.[9,43] CD spectroscopy was performed in the absence and presence of increasing amounts (1:1, 1:2, and 1:3) of this selective G-quadruplex-stabilizing ligand. As a result, the peak intensities at 264 nm significantly increased, indicative of binding and further stabilization of the parallel G-quadruplex structure (Figure 3a). Further, CD melting experiments were conducted in the absence and presence of this ligand, and Figure 3b shows the resulting CD melting profile of the 24-nucleotide G-quadruplex-forming sequence at 264 nm. As shown by the reverse sigmoidal melting curves, the oligonucleotide could form a stable G-quadruplex with a T_m of 60 ± 1 °C. Further, the presence of increasing amounts (1, 2, and 3 equiv) of ligand increased the T_m by 6, 7.5, and 11.2 °C, respectively, showing efficient binding and stabilization of the G-quadruplex by the selective G-quadruplex binder. This is in agreement with other studies with human G-quadruplexes in which different G-quadruplex ligands like porphyrin and its derivatives have shown efficient thermal stabilization.[39]

However, the confirmation of the direct binding was derived from results obtained from the ITC binding experiment performed at 25 °C. Figure 4 shows the binding isotherm of

Scheme 1. Structure of the Furan-Based Cyclic Homo-oligopeptide That Binds to the G-Quadruplex



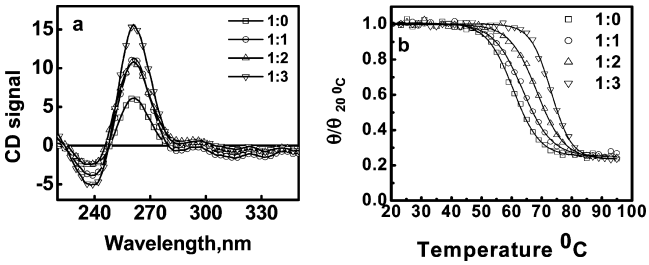


Figure 3. (a) CD spectra of the 24mer G-quadruplex-forming sequence in the absence and presence of 1, 2, and 3 equiv of the G-quadruplex selective ligand show induction of the G-quadruplex conformation. (b) CD renaturation traces of the 24mer G-quadruplex-forming sequence in the absence and presence of 1, 2, and 3 equiv of the ligand show the thermal stabilization of the G-quadruplex conformation. All experiments were performed in 10 mM sodium cacodylate containing 25 mM KCl (pH 7.0). Each experiment was repeated thrice, and average values within a 5−10% error range are reported.

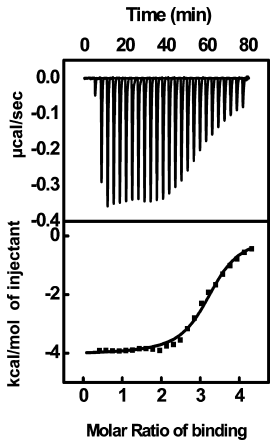


Figure 4. ITC studies show binding of the ZN-cad G-quadruplex with the selective ligand. The isotherm shows the binding interaction between the 24mer G-quadruplex-forming sequence and the ligand. The experiment was performed in 10 mM sodium cacodylate with 25 mM KCl (pH 7.0) at 25 °C. Each experiment was repeated thrice, and average values within a 5−10% error range are reported.

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the exothermic reaction, and the binding affinity was calculated to be $(2.11 \pm 0.18) \times 10^6\ M^{-1}$ with a 1:3 binding stoichiometry between the ZN-cad G-quadruplex and furan-based ligand. The ΔH value was determined to be -4.04 ± 0.5 kcal/mol. These values lie in the range of binding affinities reported for other well-established G-quadruplex and ligand interactions.[4,39] Hence, we demonstrate the formation of the ZN-cad G-quadruplex and its efficient binding and stabilization upon interaction with the previously reported G-quadruplex selective ligand. We also performed the ITC binding experiment with the 60mer duplex from the zebrafish N-cadherin proximal promoter sequence that contains putative the 24mer quadruplex site within it under similar conditions. The binding affinity of the ligand for the 60mer duplex containing the 24mer PQS was found to be $(1.0 \pm 0.1) \times 10^6\ M^{-1}$ (Figure 2 of the Supporting Information). This binding affinity was very close to the binding affinity of the ligand with the isolated quadruplex. However, when the ITC binding experiment was performed with the 60mer duplex where PQS was mutated to disrupt the quadruplex forming ability, the binding affinity was found to be $(7.0 \pm 0.5) \times 10^4\ M^{-1}$ (Figure 3 of the Supporting Information), which was almost 15 and 30 times weaker than the binding strength obtained for the ligand binding to the 60-nucleotide duplex containing the 24-nucleotide PQS and the 24-nucleotide isolated quadruplex, respectively. These data altogether suggest that the ligand binding is more selective for quadruplex structure than for duplex DNA. Additionally, we performed a Stop polymerase assay with two different sets of primers, one of which is capable of forming a G-quadruplex (G4 primers) motif similar to the ZN-cad G-quadruplex, while the other set of primers is suitably designed to interrupt the ZN-cad G-quadruplex (non-G4 primers). The data have shown that upon subsequent addition of increasing amounts of ligand in both cases, the polymerase activity in the PCR-based assay was diminished in the case of G4 primers but not in the case of non-G4 primers (Figure 4 of the Supporting Information). These results are comparable with those of previous investigations of the G-quadruplex and ligand interaction and encouraged us to attempt further exploration.

Biological Effects of G-Quadruplex Stabilization. Next, we sought to examine the biological effect of ZN-cad G-quadruplex stabilization upon ligand binding in an *in vivo* biological system. Notably, insufficient vascular growth and cardiovascular development that leads to neurodegeneration and neuronal maldevelopment are commonly reported hallmarks of perturbed N-cadherin expression.[24] Zebrafish with mutations in N-cadherin have also been identified and named as *pac* mutants and *glo* mutants.[40] They have different kinds of defects in neuronal and cardiovascular development,[24] including cardiac malformation and improper vasculogenesis. Common documented phenotypic effects upon abnormal expression of N-cadherin include thinning and mispatterning of growing vessels. Reports have suggested that the precise effects of the alteration of N-cadherin expression can also be seen in the developing intersegmental vessels (ISV) in zebrafish.[41] Hence, to measure the G-quadruplex-mediated effect on N-cadherin, we chose to observe the pattern of intersegmental vessel formation in zebrafish injected with the G-quadruplex selective ligand. For this, we selected a *Tg*(*fli1:EGFP, gata1:RFP*) zebrafish transgenic line (for details, see Materials and Methods) and injected different concentrations of the ligand into single-cell zebrafish embryos and nurtured and monitored them for 3 days. Figure 5 shows the

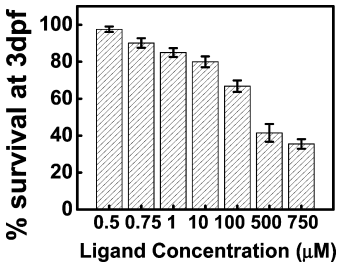

Figure 5. Survival of injected zebrafish embryos. The bar graph depicts the percentages of the surviving embryos 3 days postfertilization after injection of increasing concentrations of the G-quadruplex ligand with respect to the total number of embryos injected in each case. Each ligand concentration was represented by at least 100 embryo injections (3 nL dose) in each of the triplicate experiments. The averaged values are hence reported.

concentration-dependent survival of embryos 3 days postfertilization after the ligand had been injected. We assessed them for phenotypic effects 3 days postfertilization as the intersegmental vasculature is fully formed by 72 hours postfertilization[33,41] and any morphological changes can be optimally recorded at this stage.

At 3 days postfertilization, the embryos had developed normally with no major external malformations. The surviving embryos, however, showed certain phenotypic alterations in the intersegmental vessel patterning (shown in Figure 6). These changes were characterized by peculiarly malformed and mispatterned intersegmental vessels along with reduced blood flow, thinning of vessels, and a reduced heart rate in the affected embryos. Although in a few cases other physical changes in the anterior parts of the developing embryos were also visible, because of consistency across a larger sample population, we focused mainly on the malformations visible in the developing ISVs. The surviving population of injected embryos was then screened and scored for the presence and absence of the mentioned phenotypic changes in their intersegmental vessels. As the work of Belting and co-workers[33] showed that N-cadherin downregulation causes defects in cardiovascular development and manifests in improper or abnormal vasculogenesis, in our investigation, abnormal intersegmental vessel formation caused by N-cadherin suppression was observed. As much as 90% of the injected embryos displayed the mentioned phenotypic malformations subsequent to ligand injections. The percentage of affected embryos versus those initially injected is also presented in Figure 7. Parallel to ligand injections, a similar experiment was also performed with N-cadherin antisense morpholinos (MO). Morpholinos are specific antisense oligonucleotides that are employed to knock down the expression of a target gene in zebrafish and to help in our understanding of gene function in a reverse genetics approach. Hence, any effect produced after MO injection is regarded as the result of specific alteration and/or knockdown in the target gene. In the study presented here, we could observe phenotypic alterations in the case of MO-treated embryos in the intersegmental vasculature similar to the effect recorded with ligand-treated animals. Almost all (100%) injected embryos in this case demonstrated the altered phenotype. Also, in the case of nonspecific/control morpholino, no effect on the developing vasculature was observed. Hence, via comparison of the phenotypic changes observed above with ligand-treated and MO-treated animals, it was almost clear that such synonymous

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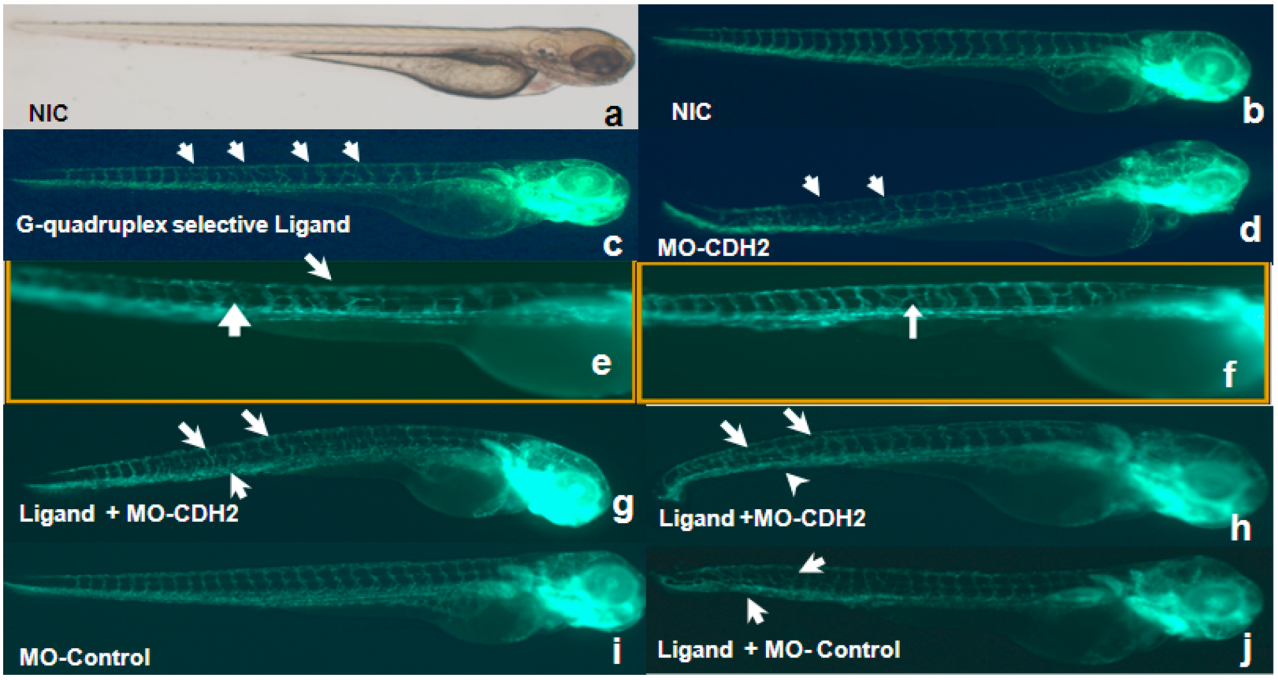

Figure 6. Phenotypic effects of G-quadruplex-stabilizing ligand injections. (a) A bright field image shows that exteriorly all animals grew without major malformations. Fluorescent images (b–j) of the phenotypic alterations observed in fish embryos 3 days postfertilization after injections with 100 μM G-quadruplex selective ligand or 20 μM morpholino against N-cadherin (MO-CDH2) or nonspecific control (MO-control). As compared to the NIC (noninjected control) (b), the ligand-treated animals (c) had mispatterened intersegmental vessels (ISV). Morpholino (MO-CDH2)-treated animals (d) also showed a similar abnormal formation of ISVs. Panels e and f show the enlarged images showing thinning and meshlike mispatterning of ISVs. Panels g and h show the additive effect of ligand and MO-CDH2 injection with severely malformed ISV development. Panel i shows no effect on ISV development in fish treated with a nonspecific/control morpholino, and co-injection of the ligand with this nonspecific morpholino (j) shows an effect similar to that of the ligand alone. All injections were performed in more than 100 embryos in each case, and representative images from three independent experiments are shown.

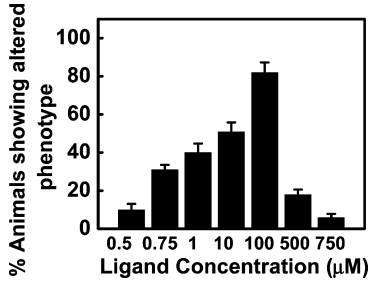

Figure 7. Extent of the effect of G-quadruplex stabilization on the phenotypic effect. This figure shows the proportions of injected embryos showing phenotypic alteration of ISV patterning as a function of an increasing concentration of the G-quadruplex selective ligand. The maximal effect was observed at a concentration of 100 μM. Beyond that concentration, fewer animals survived.

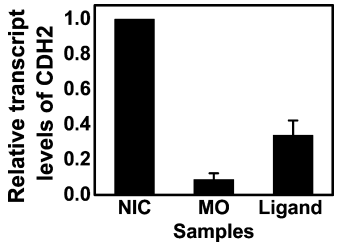

Figure 8. Real-time PCR-based quantification of ZN-cad (CDH2) mRNA. This figure shows the downregulation of CDH2 transcripts in the total mRNA isolated form embryos treated with morpholino (MO) or the G-quadruplex selective ligand as compared to β-actin 3 days postfertilization. Each sample represents total RNA isolated from 20 embryos, either from NIC or from those with a phenotypic alteration of ISV. All experiments were performed in three biological as well as technical replicates. Averaged values are hence reported.

changes were a result of the molecular perturbation of the N-cadherin inside the cells.

Molecular Effects of G-Quadruplex Stabilization with Ligand Injection. Further, to confirm if the G-quadruplex ligand could actually inhibit N-cadherin expression, the level of endogenous ZN-cad mRNA was measured. Total RNA was isolated from the affected embryos 3 days postfertilization, and real-time PCR experiments were performed with both ligand-treated and morpholino (MO)-treated animals. The results as depicted in Figure 8 show a significant downregulation of the N-cadherin or CDH2 transcripts with respect to β-actin (internal control) in ligand-treated embryos as compared to the noninjected controls (NIC), while N-cadherin or CDH2 expression was absent in the MO-treated embryos. Several previous reports with human promoter G-quadruplexes like c-MYC have established that G-quadruplex ligands could efficiently downregulate downstream gene expression.[42,43] Moreover, abolition of transcription in the case of MO injections proves the specificity of the observed effect and leads us to directly correlate the phenotypic effect with the molecular downregulation of ZN-cad.

■ CONCLUSION

For decades, the G-quadruplex has interested the research community, yet studies have been reported only for either

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unicellular or primitive organisms or mammalian cell cultures. This study, in addition to being the first report of a role of the G-quadruplex in living eukaryotic vertebrate fish, explores a novel naturally occurring G-quadruplex found in the N-cadherin gene of zebrafish. The study provides a holistic overview of the effect of ZN-cad downregulation as a result of chemical targeting with a previously reported G-quadruplex selective small molecule. The zebrafish N-cadherin G-quadruplex is found in the promoter and has been characterized in an attempt to understand the structural aspects using biophysical methods. It was understood that this motif is capable of forming a stable intramolecular G-quadruplex in a parallel topology in the presence of K^+ ions similar to the G-quadruplex found in the human c-MYC promoter. This novel G-quadruplex has a 6-4-1 loop scheme and is also thermally stable like other intramolecular DNA G-quadruplexes found in proto-oncogenes reported in the literature[44,45] as well as the human telomeric G-quadruplex. With regard to biological function, this study is the first of its kind to demonstrate the *in vivo* role of a natural G-quadruplex in a vertebrate model organism. While innumerable studies dealing with the *in vitro* and *in cellulo* demonstration of the G-quadruplex and its function have been published, this study stands out for its demonstration of the inherent phenotypic effects of chemically targeting and stabilizing the natural G-quadruplex. Hence, the study throws light on the debated existence of G-quadruplexes inside the cell.

Overall, the study provides interesting insights into G-quadruplex-mediated gene regulation in a novel system. While this is the first demonstration of the morphological manifestation of targeting a natural G-quadruplex motif in zebrafish, the same may also be optimized for artificial G-quadruplex constructs in stable fish lines. Most importantly, this study also is significant for the future development of a sophisticated chemical biology screen for characterizing G-quadruplex ligands by providing a ready handle for measuring the toxicity, efficiency, and selectivity altogether and most importantly in an animal model devoid of ethical issues. We have endeavored to understand the sophisticated convergence of significant aspects of embryonic development with G-quadruplex-mediated gene regulation, which in turn aids our understanding of developmental networks and also the study of G-quadruplexes in light of their direct functional relevance.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1−4. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: +911127666156. Fax: +9127667471. E-mail: s.sivasubbu@igib.res.in.

*Phone: +91-80-2293-2403. Fax: +91-80-2360-0529. E-mail: tushar@orgchem.iisc.ernet.in.

*Phone: +911127666156. Fax: +9127667471. E-mail: souvik@igib.res.in.

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Notes

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