Target guided synthesis using DNA nano-templates for selectively assembling a G-quadruplex binding c-MYC inhibitor

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The development of small molecules is essential to modulate the cellular functions of biological targets in living system. Target Guided Synthesis (TGS) approaches have been used for the identification of potent small molecules for biological targets. We herein demonstrate an innovative example of TGS using DNA nano-templates that promote Huisgen cycloaddition from an array of azide and alkyne fragments. A G-quadruplex and a control duplex DNA nano-template have been prepared by assembling the DNA structures on gold-coated magnetic nanoparticles. The DNA nano-templates facilitate the regioselective formation of 1,4-substituted triazole products, which are easily isolated by magnetic decantation. The G-quadruplex nano-template can be easily recovered and reused for five reaction cycles. The major triazole product, generated by the G-quadruplex inhibits c-MYC expression by directly targeting the c-MYC promoter G-quadruplex. This work highlights that the nano-TGS approach may serve as a valuable strategy to generate target-selective ligands for drug discovery.
Small molecules are fundamental probes for studying biological systems. Considering the dynamic nature of the biological macromolecules, the development of small molecules that can interact with biological targets with high affinity and selectivity has always been challenging and time consuming. Target Guided Synthesis (TGS) is a powerful approach to discover novel and selective small molecule binders for biological targets by combining the synthesis and screening into a single step. It uses a biological target to synthesize its own best binders from a series of small molecule fragments, functionalized with complementary reacting groups. In a pioneering work, Sharpless and co-workers developed in situ click chemistry, a kinetically controlled TGS approach in which the enzyme acetylicholinesterase was used as the target to assemble its potent small molecule inhibitors from a pool of azide and alkyne building blocks. In TGS, the azide and alkyne fragments undergo Huisgen 1,3-dipolar cycloaddition (Click reaction) in the presence of a catalytic target that brings the azide and alkyne building blocks into close proximity with correct spatial orientation to generate one of the triazole regioisomers in the absence of any metal catalyst. Therefore, small molecules identified by TGS are expected to show high binding affinity as well as specificity for the target as they are synthesized by a specific reaction in which the active site of the biological target controls the assembly of the best binding fragments. The majority of TGS approaches, reported to date, use various enzymes as the target to assemble their potent inhibitors. Only two examples are reported so far using nucleic acids as the targets. However, these solution phase TGS methods have some limitations like poor isolation of the lead compounds from the reaction mixture comprising the target and fragment library and lack of reusability of the target for multiple rounds of templated synthesis.

We anticipated that the use of magnetic nanoparticle supported DNA sequences as the targets could overcome such shortcomings: the in situ lead compounds could be easily isolated by simple magnetic decantation and the DNA templates could be easily recovered and recycled. In addition, the DNA sequences immobilized on the nanoparticle surface would greatly enhance the rate of reaction between small molecule fragments by providing a larger surface area with more numbers of localized DNA templates.

In the present study, we describe an azide-alkyne cycloaddition based TGS approach using c-MYC G-quadruplex and control duplex DNA monolayers assembled on gold-coated magnetic nanoparticles as the templates. The c-MYC G-quadruplex present in the NHE III1 region upstream of the P1 promoter is believed to be involved in the complex regulation of ruplex present in the NHE III1 region upstream of the P1 control duplex DNA monolayers assembled on gold-coated nanoparticles (Fe3O4 MNPs) have shown good promise in biological applications as they are non-toxic, naturally available and easily synthesizable with superior magnetic and chemical properties. Gold coating on these nanoparticles improves the stability of the nanoparticles in solution, makes them biocompatible and provides an outer layer for covalently tethering thiolated biomolecules on nanoparticle surfaces by chemisorption. The Au@Fe3O4 nanoparticles were prepared by citrate reduction of Fe3O4 nanoparticles by transmission electron microscopy (TEM) and atomic force microscopy (AFM) experiments. Both the Fe3O4 and Au@Fe3O4 were nearly spherical and relatively mono-dispersed with a mean diameter of 11 and 15 nm, respectively (Supplementary Fig. 1). The G4-Au@Fe3O4 nanoparticles were prepared by citrate reduction of Fe3O4 nanoparticles (see Methods) and the dispersion of Au@Fe3O4 NPs in 100 mM Tris-KCl buffer (pH 7.4) displayed a SPR absorption band near 535 nm (refs 29–31). The DNA linked Au@Fe3O4 NPs (G1•Au@Fe3O4 and dsDNA•Au@Fe3O4) also showed characteristic SPR bands of Au at 535 nm. The SPR of Au@Fe3O4 and dsDNA•Au@Fe3O4 dispersions absorb at 270 and 280 nm, respectively, suggesting DNA modification on the MNPs (Supplementary Fig. 1b).

The surface functionalization of the nanoparticles with thiolated c-MYC G4 DNA was further confirmed by CD spectroscopy. The CD spectrum of G4•Au@Fe3O4 nano-template exhibited a positive peak at 260 nm and a negative peak at 240 nm, which indicates that it retains the parallel conformation of the c-MYC quadruplex (Fig. 1c). The DNA-linked nanoparticles exhibited paramagnetic properties of Fe3O4 MNPs (Supplementary Fig. 1). Then we characterized the DNA-linked nanoparticles by transmission electron microscopy (TEM) and atomic force microscopy (AFM) experiments. Both the Fe3O4 and Au@Fe3O4 were nearly spherical and relatively mono-disperse with a mean diameter of 11 and 15 nm, respectively (Supplementary Fig. 1). The EDX spectra of Au@Fe3O4 NP indicated the presence of both Fe and Au in the particles (Supplementary Fig. 1). The G4•Au@Fe3O4 MNPs also preserved the near spherical shape of the NPs (Fig. 1d). The attachment of thiol-DNA onto Au@Fe3O4 MNPs was further assessed by β-mercaptoethanol (β-ME) mediated thiol exchange reaction that displaced the surface bound c-MYC G4 DNA, visualized by Native PAGE analysis (Supplementary Fig. 2).

**Design and synthesis of azide and alkyne building blocks.** To generate high-affinity G-quadruplex ligands, a library of azide and alkyne building blocks was prepared (Fig. 2). The alkynes 1a, 1b and 1c were derived from carbazole, a rigid heteroaromatic ring system present in numerous natural products and pharmacologically active compounds. The alkine 1a was prepared in good overall yield from the commercially available carbazole using mono-iodination, N-arylation followed by amide coupling and Sonogashira reaction (Supplementary Fig. 3). Alkyne 1b was simply prepared from the mono-iodo carbazole by Sonogashira coupling (Supplementary Fig. 4) and alkyne 1c was derived from mono-iodo carbazole by N-alkylation followed by Sonogashira coupling (Supplementary Fig. 5). The reason for selecting carbazole ring system is that it can interact with the G-quadruplex DNA through π-π stacking interactions with the external G-quartets. Our azide library contains a range of aliphatic and aromatic azides that includes amines (2 and 7), alcohols (4 and 7), simple phenyl ring (5), aromatic aldehyde (8), ester (9) and carboxamide with amine side chains (11). It also contains amino acid and nucleoside derivatives such as prolinamide (10) and guanosine (12). These azide building blocks may interact with G-quadruplex using π-π stacking interactions by the aromatic ring systems and electrostatic interactions.
interactions by the protonable side chains. The synthesized azide and alkyn library would generate 66 theoretically possible triazole products including 1,4 and 1,5 regioisomers that may interact with the target G-quadruplex.

**G₄•Au@Fe₃O₄ MNPs templated synthesis.** We then evaluated the ability of G₄•Au@Fe₃O₄ to template the metal free 1,3-dipolar cycloaddition. In a typical experiment, a 4:1 mixture of azide and alkyn building blocks 1–12 were stirred with c-MYC G₄•Au@Fe₃O₄ NPs in Tris-KCl buffer (100 mM, pH 7.4). After 6 days, the reaction mixture was treated with the denaturing agent lithium chloride (LiCl) and heated to 65°C to unfold the DNA-quadruplex (Supplementary Fig. 6). The HPLC chromatogram of the supernatant collected after magnetic separation of DNA-MNPs showed an inseparable complex mixture of compounds with overlapping peaks (Supplementary Fig. 6). Subsequently, the purification protocol was modified by simply heating the mixture at 65°C without adding LiCl (ref. 37). However, the *in situ* lead compounds could not be identified as overlapping peaks were obtained in HPLC (Supplementary Fig. 6).

We then modified the separation technique to easily identify and isolate the *in situ* leads. As the resultant triazole products are expected to bind the G-quadruplex magnetic nano-template, the separation technique was improved to (i) detect each generated triazole leads, the complex analysis of the reaction mixture in an optimum time and (ii) recycle the DNA template for successive azide-alkyne cycloadditions. After incubating the mixture of azide and alkyn fragments 1–12 with the G₄•Au@Fe₃O₄ MNPs for 6 days, the MNPs along with the newly formed bound products were separated from the reaction mixture using an external magnet and washed three times with 100 mM Tris-KCl buffer. The unreacted fragments remained in the supernatant. The MNPs were then resuspended in 100 mM Tris-KCl buffer, heated to 65°C to release the products bound with the G-quadruplex nano-template. The DNA-MNPs were separated instantly by magnetic decantation and the supernatant was collected and characterized by HPLC and ESI-MS analysis (Fig. 3, Supplementary Methods).

We observed the formation of three triazole compounds (Tz 1–3 in 62:7:31 percentage ratio) in the presence of the c-MYC G₄•Au@Fe₃O₄ NPs (Fig. 4a). For a comparison, we have performed the TGS reaction with conventional c-MYC G-quadruplex DNA sequence (5′-G₄A₃G₄A₃T₃G₄A₃-3′), that yielded essentially similar lead compounds which were identified in ESI-MS analysis but overlapping peaks were obtained in HPLC chromatogram. This result validates the use of G₄•Au@Fe₃O₄ MNPs as an alternative template to the homogeneous G-quadruplex DNA for the templated synthesis of G-quadruplex ligands. This method thus provides advantages over the conventional solution phase TGS as the resulting products can be efficiently analysed and separated.

Simultaneously, the reaction was performed in the presence of MNPs functionalized with duplex DNA (dsDNA•Au@Fe₃O₄) (Supplementary Fig. 8) as the control and only one triazole compound Tz 2 (combination of alkyn 1a and azide 3) was detected in MS-HPLC analysis (Fig. 4b). This protocol thus validates products Tz 1 (alkyn 1a and azide 11) and Tz 3 (alkyne 1a and azide 7) as the selective *in situ* lead compounds for the G-quadruplex DNA target (Fig. 4c,d). Compound Tz 2 non-specifically binds to both quadruplex and duplex DNA. It is worth noting that all the lead compounds are derived from alkyn 1a, which indicates that the DNA templates preferentially select alkyn 1a over alkyn 1b and 1c. However, the solubility of these fragments in aqueous buffer may play a role in their selection. Other possible products were not formed or detected in considerable amount indicating that the corresponding azides may not be optimally oriented by the c-MYC G-quadruplex template to react with the alkyn partners. Moreover, the preferential formation of Tz 1 suggests that the c-MYC G-quadruplex selected the azide 11 due to its aromatic carboxamide group and positively charged amine side chain that facilitates π–π stacking with additional electrostatic interactions with the quadruplex DNA.

We then analysed the product distribution of the reaction mixture with G₄•Au@Fe₃O₄ template at two different time points. By stirring the reaction for 2 days, the major lead Tz 1 and
Tz 2 (exclusively formed by duplex DNA) were obtained in a percentage ratio of 65:35 (Supplementary Fig. 7). After 4 days, the formation of Tz 1 was increased compared to Tz 2 (78:22), while the other lead Tz 3 was not detected in the mixture (Supplementary Fig. 7). The formation of Tz 2 was not significantly increased by the G-quadruplex template with time suggesting...

Figure 2 | Design of building blocks for templated cycloaddition. (a) G₄•Au@Fe₃O₄ NP templated azide-alkyne cycloaddition. (b,c) Structures of carbazole alkynes 1a–c and azides 2–12.

Figure 3 | Selective cycloaddition by G₄•Au@Fe₃O₄ nano-template. Schematic representation of templated cycloaddition between selective azide and alkyne partners using G₄•Au@Fe₃O₄ nanoparticles.

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HPLC analysis using G4
c-MYC
to conclude that the
formed by
c-MYC
partners on short time scales.

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promoted the preferential formation of

separation protocol; HPLC analysis using (NATURE COMMUNICATIONS| 8:16103 | DOI: 10.1038/ncomms16103 | www.nature.com/naturecommunications
Supplementary Fig. 11).

Tz 1 formation of the 1,4-regioisomer of
alkyne 1a
NPs promote the cycloaddition between the proximally oriented
DNA. However, the formation of
weaker binding affinity of
Tz 1 as the potent lead
compound and it could not bind other weak alkyne-azole
partners on short time scales.

To determine the regioselectivity of the triazole product Tz 1
formed by c-MYC G4●Au@Fe3O4 NP templated synthesis, we
have carried out the cycloaddition of alkyne 1a and azide 11
under both thermal and Cu(I) catalysed conditions (Supplementary
Fig. 9). The Cu(I) catalysed cycloaddition gave the
1,4-substituted triazole product and the thermal Huisgen
cycloaddition between the corresponding azide and alkyne
generated both the 1,4- and 1,5-substituted regioisomers. Both
the compounds have identical mass and the 1,5-substituted
tripazole product has a slightly lower retention time. By comparing
the HPLC retention times, we have observed that the
triazole product has a slightly lower retention time. By comparing
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cycloaddition from their respective alkyne/

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Figure 4 | Monitoring of G4●Au@Fe3O4 mediated target guided synthesis. HPLC chromatogram of the supernatant collected following the modified
separation protocol; HPLC analysis using (a) G4●Au@Fe3O4 MNPs and (b) dsDNA●Au@Fe3O4. (c) ESI-MS analysis of the three fractions collected from the
HPLC analysis using G4●Au@Fe3O4 reveals the formation of compounds Tz 1, Tz 2 and Tz 3. (d) The molecular structures of compounds Tz 1, Tz 2 and Tz 3.

Recovery and reuse of the c-MYC DNA nano-template. Next, we
investigated the ability of the magnetically recovered c-MYC
G4●Au@Fe3O4 nanoparticles to template the Huisgen cycloaddition
using the same azide-alkyne fragments 1–12. After 6 days, the
recovered c-MYC MNPs provided the triazole products Tz 1–3
with nearly similar product distribution (63:6:31) as the freshly
prepared c-MYC G4●MNPs (Supplementary Fig. 12). The product
distribution was found to be similar in the subsequent reaction cycle.
In the third cycle, Tz 1 and Tz 2 were obtained while the formation of
Tz 3 (the other G4-binding ligand) was not observed in the HPLC
chromatogram. In the fourth and fifth cycle, Tz 1 was exclusively
formed, indicating high recycling ability of the nano-template
(Fig. 5). It also suggests that the recovered nano-template maintains
its templating activity and facilitates the formation of its strong
binder from the pool of azide and alkyne fragments.

Tz 1 selectively binds c-MYC G-quadruplex over duplex DNA.
To validate the compound Tz 1 as the best binding ligand for
c-MYC G-quadruplex DNA over duplex DNA, we have
synthesized the three in situ lead products Tz 1–3 by the Cu(I)
catalysed azide-alkyne cycloaddition from their respective alkyne/azide precursors (Supplementary Figs 13 and 14) and compared
their stabilization potential using Förster resonance energy transfer
(FRET) based melting assay. We have used 5’-FAM and 3’-
TAMRA-labelled c-MYC G-quadruplex (100 nM) and a control

weaker binding affinity of Tz 2 for the c-MYC G-quadruplex
DNA. However, the formation of Tz 1 was greatly accelerated by
G4 DNA template with increasing time. These observations lead
us to conclude that the c-MYC G4●Au@Fe3O4 nano-template
promoted the preferential formation of Tz 1 as the potent lead
compound and it could not bind other weak alkyne-azole
partners on short time scales.

To determine the regioselectivity of the triazole product Tz 1
formed by c-MYC G4●Au@Fe3O4 NP templated synthesis, we
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1,4-substituted triazole product and the thermal Huisgen
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generated both the 1,4- and 1,5-substituted regioisomers. Both
the compounds have identical mass and the 1,5-substituted
tripazole product has a slightly lower retention time. By comparing
the HPLC retention times, we have observed that the c-MYC
G4●Au@Fe3O4 NPs templated the 1,3-dipolar cycloaddition of
alkyne 1a and azide 11 leading to the formation of the identical
product as in the Cu(I) catalysed reaction (Supplementary
Fig. 10). These results illustrate that the c-MYC G4●Au@Fe3O4
NPs promote the cycloaddition between the proximally oriented
alkyne 1a and azide 11 in a regioselective manner resulting in the
formation of the 1,4-regioisomer of Tz 1 (relative yield 44%;
Supplementary Fig. 11).
Both C-MYC compound Tz 3 DNA (Supplementary Fig. 15). The major lead compound these carbazole derivatives are environmentally sensitive probes titration (Fig. 6d–i). Fluorescence binding titration revealed that fluorescence experiments further extended aromatic system can selectively target G-quadruplex DNA. The triazole compound Tz 1 showed better stabilization potential (ΔT_m) towards c-MYC G-quadruplex DNA over dsDNA compared to other triazole derivatives.

FRET competition experiment was performed to evaluate the selectivity of Tz 1 for the c-MYC G-quadruplex DNA over duplex DNA (Supplementary Fig. 15). The ΔT_m value of ligand Tz 1 for the c-MYC quadruplex showed a negligible change in the presence of 20 equivalent excess of dsDNA, indicating high selectivity of Tz 1 for the quadruplex over duplex DNA. In addition, the circular dichroism (CD) spectra for the c-MYC quadruplex show that Tz 1, even at high concentrations, did not perturb the parallel conformation of c-MYC G-quadruplex DNA (Supplementary Fig. 16).

As the carbazole derivatives are fluorescent, the binding affinities of the in situ lead compounds Tz 1, 2 and 3 for both quadruplex and duplex DNA were investigated by fluorescence titration (Fig. 6d–i). Fluorescence binding titration revealed that these carbazole derivatives are environmentally sensitive probes for the G-quadruplex. The major lead compound Tz 1 exhibited a selective fluorescence enhancement (3.7-fold) upon binding with G-quadruplex with a 33 nm blueshift, whereas it barely showed any enhancement upon titration with duplex DNA. Compound Tz 2 showed enhanced fluorescence intensity (2.5-fold) with a significant blueshift of ~40 nm in the presence of both c-MYC G-quadruplex and dsDNA. The significant blueshift observed for compounds Tz 1 and Tz 2 containing the electron donor and acceptor motifs, indicated their binding with the hydrophobic sites of the DNA. In comparison, Tz 3 produced a slight wavelength change (7 nm redshift) with a 1.9-fold increase in fluorescence intensity upon addition of c-MYC G-quadruplex. The fluorescence experiments further indicated that Tz 1 is selective for the c-MYC quadruplex DNA compared to Tz 2 and Tz 3 and it has high affinity for MYC quadruplex (K_D = 0.17 ± 0.01 μM) (Table 1, Supplementary Fig. 17).

The FRET melting analysis and fluorescence titration results are consistent with the results obtained in c-MYC G•Au@Fe3O4 NP templated target guided synthesis approach. The major lead compound Tz 1 containing two protonable tertiary amine side chains strongly interacts with quadruplex compared to the second lead compound Tz 3 that contains one amine side chain. In addition, the minor lead Tz 2 lacking the aromatic carboxamide group was formed as a major product in the presence of duplex DNA template, which further indicates that compounds with extended aromatic system can selectively target G-quadruplex over duplex DNA. The triazole compound Tz 1 that showed high affinity for the c-MYC G-quadruplex DNA, was selected for carrying out subsequent biological assays to highlight the importance of this methodology to discover important leads for G-quadruplex specific drug development.

**Downregulation of c-MYC expression by Tz 1.** We have also assessed the biological activity of Tz 1 in HCT116 colorectal
adenocarcinoma cancer cell line. HCT116 has been reported as a model cell line for monitoring the c-MYC expression in living cells\(^4\). Fluorescence microscopic images of Tz 1 treated HCT116 cells reveals that Tz 1 permeates cell and nuclear membranes and preferentially localizes in the nuclei of the cells (Fig. 7a–c). We then evaluated the short-term growth inhibitory activity of Tz 1 in HCT116 cell line using MTT assay. Compound Tz 1 showed strong inhibitory activity on the proliferation of HCT116 cells in a dose-dependent manner, with an IC\(_{50}\) value of 2.1 ± 0.1 μM.

Next, we have evaluated the effect of Tz 1 on the transcriptional and the translational level of c-MYC gene. qRT-PCR analysis showed that Tz 1 reduced the transcription of c-MYC gene by 44 and 79% (normalized to GAPDH expression) at 1 and 2 μM concentrations, respectively, compared to the untreated cells. Western blot analysis corroborated the inhibitory activity of Tz 1 on the c-MYC expression by suppressing the c-MYC protein levels by 39 and 74% at 1 and 2 μM treatment, respectively. The expression of GAPDH in HCT116 cells remained unaltered by Tz 1 (Fig. 7f,g).

To further evaluate whether the observed c-MYC inhibition by Tz 1 was dependent on the ligand mediated G-quadruplex stabilization in the c-MYC promoter region, CA46 Burkitt’s lymphoma cell line was used to perform the exon-specific assay\(^4\). In CA46 cell line, the transcription of MYC exons are differentially regulated; exon 1, which generally remains untranslated, are transcribed under the control of G-quadruplex forming NHE III region. Whereas exon 2, which is predominantly responsible for MYC protein expression, is independent of G-quadruplex regulation due to the
translocation occurred between the chromosome 8 (MYC gene) and 14 (IgH heavy chain gene). Thus the transcription of exon 2 mainly represents the MYC expression from the G-quadruplex-lost enhancer element of IgH heavy chain. This translocational alteration of the MYC gene locus renders the MYC protein expression unaffected to the inhibition by G-quadruplex stabilization. Using this model, we have demonstrated the G-quadruplex stabilizing ability of Tz 1 by independently monitoring the two MYC mRNA products produced within the CA46 cell line upon ligand treatment.

The qRT-PCR assay revealed that Tz 1 significantly induced exon-specific downregulation of MYC expression in a dose-dependent manner. It caused a noteworthy inhibition of exon 1 transcription (95% at 1 μM and 98% at 2 μM...
concentration), which is regulated by G-quadruplex formation, while transcription from exon 2 remains unaffected after 24 h treatment with Tz 1 (Fig. 7h). Thus, on the basis of the observed exon-specific effect of Tz 1, we can conclude that Tz 1 binds and stabilizes the G-quadruplex structure in c-MYC PI promoter and specifically reduces c-MYC expression in cancer cells. It is well documented that c-MYC plays a critical role in the regulation of the cellular fate between apoptosis and necrosis and therefore quadruplex interacting molecule that can repress c-MYC expression can drive cells towards apoptosis.43 We investigated the ability of Tz 1 to induce cell apoptosis in HCT116 cells by flow cytometry. Tz 1 induced apoptotic cell death in a dose-dependent manner, as demonstrated by the dual staining with propidium iodide (PI) and Annexin V-FITC. The population of the apoptotic cells was increased significantly in the presence Tz 1, while there was no remarkable increase of necrotic cells upon 24 h treatment (Supplementary Fig. 18). The observed cellular effect of the lead compound Tz 1 is likely due to its interaction with the G-quadruplex DNA in the c-MYC promoter region. It can be possible that Tz 1 can interact with other quadruplexes among which we have only demonstrated its interaction with c-MYC promoter quadruplex DNA. However, this is not surprising as many drugs, even when approved; expectedly do not have all the required properties for their appropriate function. This also reinstates the importance of quadruplex ligands. This methodology using DNA nano-templates for metal free Huisgen cycloaddition offers several advantages: First, it reduces the time required for the conventional laborious chemical synthesis of potent candidates and their biological binding/screening assay. Second, it provides a large surface with more number of local DNA catalytic sites for better interaction of the reacting fragments on the nano-surface. Thirdly, it enables efficient isolation of the in situ lead compound by simple magnetic decantation and lastly, it prevents the wastage of commercial DNA sequences by allowing the recovery and reuse of DNA magnetic nano-templates. This innovative approach has been used for a model library of azide and alkyne building blocks to obtain two 1,4-triazole lead compounds (out of 33 possible triazole products of 1,4-regiochemistry) for the G-quadruplex target. The major lead compound shows high binding affinity for c-MYC G-quadruplex DNA over duplex DNA, localizes exclusively into the nucleus, inhibits the c-MYC expression by targeting the c-MYC promoter G-quadruplex and induces apoptosis in HCT116 cells. We anticipate that this proof-of-concept study would pave the way for the design and fabrication of biomolecule immobilized magnetic nano-constructs, exhibiting potentially important impacts both in biomanotechnology and biomedical research.

Discussion

In this work, we have established the use of magnetic nanoparticle-linked c-MYC G-quadruplex as a template for covalently assembling potent azide-alkyne partners to generate high-affinity quadruplex ligands. This methodology using DNA nano-templates for metal free Huisgen cycloaddition offers several advantages: First, it reduces the time required for the conventional laborious chemical synthesis of potent candidates and their biological binding/screening assay. Second, it provides a large surface with more number of local DNA catalytic sites for better interaction of the reacting fragments on the nano-surface. Thirdly, it enables efficient isolation of the in situ lead compound by simple magnetic decantation and lastly, it prevents the wastage of commercial DNA sequences by allowing the recovery and reuse of DNA magnetic nano-templates. This innovative approach has been used for a model library of azide and alkyne building blocks to obtain two 1,4-triazole lead compounds (out of 33 possible triazole products of 1,4-regiochemistry) for the G-quadruplex target. The major lead compound shows high binding affinity for c-MYC G-quadruplex DNA over duplex DNA, localizes exclusively into the nucleus, inhibits the c-MYC expression by targeting the c-MYC promoter G-quadruplex and induces apoptosis in HCT116 cells. We anticipate that this proof-of-concept study would pave the way for the design and fabrication of biomolecule immobilized magnetic nano-constructs, exhibiting potentially important impacts both in biomanotechnology and biomedical research.

Methods

Chemical synthesis. Detailed procedures for the synthesis of all new compounds and their characterization are provided in Supplementary Methods (For 1H and 13C NMR spectra of all compounds, Supplementary Methods and Supplementary Figs 19–36).

Synthesis of Fe3O4 nanoparticles. The Fe3O4 nanoparticles were prepared by co-precipitation technique. This method uses ferric and ferrous ions in a 1:2 molar ratio in basic solutions. An aqueous solution of FeCl3 (4 ml, 1 M) and FeCl2 (1 ml, 2 M in 2 M HCl) was mixed and added into diluted NH4 solution (50 ml, 0.7 M). The reaction mixture was stirred for 30 min at RT. Then, the precipitate was isolated by magnetic decantation, stirred with distilled HClO4 (30 ml, 2 M) and then collected by centrifugation. The residue was finally made up to 50 ml with deionized water.

Synthesis and characterization of AuFe3O4 nanoparticles. An aqueous solution of HAuCl4 (2.26 ml, 2.0 mg ml−1) was mixed with 15.75 ml deionized water and then heated to boiling. Then, 0.75 ml of Fe3O4 nanoparticles solution was added into the reaction mixture followed by the addition of sodium citrate (0.75 ml, 80 mM) with continuous stirring. The colour of the solution was gradually changed from brown to burgundy. The reaction mixture was heated to 85°C under stirring for 5 min. After cooling, the solution was sonicated for 15 min, and then AuFe3O4 NPs were collected using a magnetic, washed three times and dispersed in pure water.

The prepared AuFe3O4 NPs were characterized in 100 mM Tris-KCl buffer (pH 7.4) by ultraviolet-visible absorption spectroscopy (Cary Win 300). The UV-visible spectrum was measured using quartz cuvette of path length 1 mm at 25°C, CD spectroscopy (Jasco J-815), TEM (JEOL 1200 EX electron microscope) and atomic force electron microscopy (NT-MDT instrument in semicontact mode with a resonance frequency of 120 kHz).

Synthesis of DNA coated AuFe3O4 NPs. All buffers and solutions were bubbled with N2 and degassed to avoid oxidative dimerization of thiolated DNA sequences into disulfide dimers. Thiolated DNA attached nanoparticle probes were synthesized by mixing an aqueous dispersion of AuFe3O4 nanoparticles (100 µl) with 100 µl 5′-thiol-capped c-MYC G-quadruplex (oligonucleotide concentration is 100 µM in 100 mM Tris-KCl buffer, pH 7.4). After standing for 16 h, the nanoparticles were separated using magnet and washed with 100 mM Tris-KCl buffer (pH 7.4). The volume of the nanoparticle solution was made up to 100 µl with buffer. Duplex DNA was also attached on the nanoparticle surface by following the same protocol. The HPLC purified thiol-capped DNAs were supplied by Sigma-Aldrich. The sequences of the DNAs are as follows: dsDNA: [ThiC6]GGGGAGGGTGCAAAAATTGTG

β-Mercaptoethanol mediated thiol exchange reaction. AuFe3O4 nanoparticle was surface functionalized with different concentrations of quadruplex DNA (10–50 µM). The DNA attached nanoparticles were then separated by a magnetic decantation and washed 3 to 4 times with Tris-KCl buffer, pH 7.4. β-Mercaptoethanol (β-ME) was added (final concentration 12 mM) to the oligonucleotide-modified nanoparticles in 100 mM Tris-KCl buffer (pH 7.4). After 18 h incubation at room temperature with intermittent shaking, the solutions containing the displaced oligonucleotides were separated from the AuFe3O4 nanoparticles using an external magnet. The supernatant was then subjected to Native PAGE to determine the surface functionalization of nanoparticles with thiolated oligonucleotides.

General procedure for G4-AuFe3O4 templated cyclodaddition. To a suspension of G-quadruplex nano-template G4·AuFe3O4 (10 µl) in 20 µl Tris-KCl buffer (100 mM, pH 7.4), alkynes 1a–c followed by azides 2–12 were added. The stock solutions of all alkynes and azides were prepared in 5% DMSO buffer, pH 7.4 and then serially diluted in 100 mM Tris-KCl buffer. Final reaction concentrations were as follows: alkyne (0.6 mM), azide (2.4 mM). The mixture containing the nano-template and azide-alkyne building blocks was stirred at room temperature. The cyclodaddition products were identified by optimizing the separation protocols. After 2, 4 and 6 days of reaction, the G4·AuFe3O4 NPs were separated from the reaction mixture using a magnet and washed thrice with 100 mM Tris-KCl buffer, pH 7.4 (100 µl) to remove the unreacted starting materials. Subsequently, the nano-template was dispersed in 100 mM Tris-KCl buffer, pH 7.4 (50 µl) and the dispersion was then heated for 5 min at 65°C. The nano-template was again separated instantly by magnetic decantation and the supernatant was analysed by HPLC. The HPLC fractions corresponding to different peaks were identified by ESI-MS spectroscopy.

Control experiment using duplex DNA functionalized nanoparticles (dDNA-AuFe3O4 NPs) was performed using similar experimental conditions. The HPLC analysis was carried out using 5.0 µm ODS2 reverse phase column (4.6 × 250 mm) using 254 nm detection wavelength. Flow rate was 0.5 ml min−1 CH3CN/CH3COOH (90:10) in 0.1% TFA over 20 min.

FRET melting analysis. FRET melting experiments were carried out with a 100 nM oligonucleotide target in 60 mM potassium cacodylate buffer, pH 7.4. All HPLC purified dual labelled DNA oligonucleotides were purchased from Sigma-Aldrich. 5′-FAM- and TAMRA-labelled DNA sequences were annealed at a concentration of 200 nM by heating at 95°C for 5 min followed by gradual cooling to room temperature at a controlled rate of 0.1°C min−1. Dual fluorescently labelled DNA oligonucleotides used in these experiments are: c-MYC: 5′-FAM- GGAGGGGTGGGGTGAGGTGGGG -TAMRA-3′

dDNA: 5′-FAM- CAAAAATTTTTGCAAAAAATTTTGTGCAAAAATTGTG-3′
FRET based screening assay was performed on a real-time PCR apparatus (Light Cycler 480 II System) by incubating the dual labelled DNA oligonucleotides (100 nM) with 1 μM of each triazole derivatives Tx 1–3 separately for 1 h. Fluorescence measurements were taken with an excitation wavelength of 483 nm and a detection wavelength of 533 nm at intervals of 1 °C over the range of 37–95 °C. Melting temperatures were calculated using Origin Pro 8 data analysis. Titration experiments were performed by adding different concentrations of Tx 1, Tx 2 and Tx 3 separately (0.3, 0.5, 0.8, 1.0, 2.0, 3.0, 5.0, 7.0 and 10.0 μM) to 100 nM solution of dual labelled c-MYC and duplex DNA in 60 mM potassium cacodylate buffer (pH 7.4). In FRET competition experiment, the concentration of Tx 1 was kept at 1.0 μM in all sets. The competitor duplex DNA was added to 100 nM c-MYC-G quadruplex sequence at final concentration of 100 nM, 500 nM, 10 μM, and 2.0 μM.

Circular dichroism spectroscopy. CD spectra were recorded on a Jasco J-815 spectrophotometer using a 1 mm path-length quartz cuvette. Aliquots of ligand Tx 1 were added in increments to pre-anneled c-MYC-G quadruplex DNA (5′-GCGGAGGTTGGGAGGGTTGGG-G3′) in Tris-KCl (100 mM) buffer at pH 7.4. The DNA concentration used was 10 μM. The CD spectra represented an average of three scans and were smoothed and zero corrected. Final analysis of the data was carried out using Origin 8.0.

Fluorometric titration. The fluorescence spectra were recorded on a Horiba Fluoromax 3 instrument at 25 °C in a 10 mm path-length quartz cuvette with filtered 100 mM Tris-KCl buffer (pH 7.4). Fluorescence titrations were performed with successive addition of the DNA solution into the 1 μM ligand solution. The binding constants were calculated using the following Hill equation (1) (with Origin Pro 8.0):

\[
F = F_0 + \frac{(F_{\text{max}} - F_0) [\text{DNA}]}{K_D + [\text{DNA}]}
\]

where \(F\) is the fluorescence intensity, \(F_{\text{max}}\) is the maximum fluorescence intensity, \(F_0\) is the fluorescence intensity in the absence of DNA, and \(K_D\) is the dissociation constant.

In this experiment, following DNA sequences were utilised: c-MYC: 5′-GGGAGGGTTGGGAGGGTTGGG-G3′
ddDNA: 5′-CAAAAATTTTGGCAAAAAATTTT-G3′

Cell culture and viability assay. HCT116 cell line (ATCC) was a kind gift from Dr Susanta Roychoudhury, Indian Institute of Chemical Biology (Kolkata). CA46 cell line was procured from AICC. The cells were cultured at 37 °C in 5% CO₂ atmosphere and maintained in DMEM (Dulbecco’s modified Eagle Medium, Himedia Cell Culture) atmosphere and maintained in DMEM (Dulbecco’s modified Eagle Medium, Himedia Cell Culture) atmosphere and maintained in DMEM (Dulbecco’s modified Eagle Medium, Himedia Cell Culture) atmosphere.

Fluorescent microscopy. The HCT116 cells were plated in six-well plates and treated with 5 μM Tx 1 for 8 h. After the incubation, the cells were washed with PBS and fixed for 12 min with 1:1 acetone/methanol at −20 °C. The cells were rinsed thrice in PBS, stained with DAPI and kept at room temperature in the dark for 10 min. The cells were then washed twice with PBS, examined and immediately photographed under a fluorescence microscope (Evos FL cell imaging system, ThermoFisher Scientific Ltd.).

gQ-PCR analysis. Colorectal cancer cell line HCT116 cells were treated with two different concentrations (1.0 and 2.0 μM) of triazole ligand Tx 1. Total RNA from treated and control cells were prepared using the trizol kit according to the manufacturer’s protocol (ThermoFisher Scientific, catalogue number 15596081). Reverse transcription was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, catalogue number 4368814) following the kit manual. The prepared cDNA was amplified by PCR using Power SYBR Green PCR Master Mix (ThermoFisher Scientific, catalogue number 4367659) with the forward and reverse primers in Light Cycler 480 II (Roche). Primers used in the amplification of c-MYC and GAPDH gene: c-MYC (forward): 5′-CTGCCACAGGAGGAGGAC-3′
c-MYC (reverse): 5′-GGGAGGAGGCTTCTTCTTCT-3′
GAPDH (forward): 5′-GAGGGGCAGATCTTGTGTA-3′
GAPDH (reverse): 5′-CACACCGACCTCTACACATT-3′
The PCR mixture (20 μl) contained 500 nM of each primer, 5 μl of cDNA template and 10 μl of SYBR green mix and the rest of the volume is made up with nuclease free water. The thermal cycling conditions were 10 min at 95 °C and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The relative mRNA expression was determined by the arithmetic calibrator (2^ΔΔCT) and the difference in c-MYC expression was expressed as fold changes.

Western blot analysis. After 24 h incubation of HCT116 cells with the above mentioned concentrations of Tx 1, cells were washed once with PBS (pH 7.4) and lysed with ice-cold cell lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA in 0.5% Triton X-100). Cell lysate was collected from the treated and untreated cells, and the total protein content was estimated by Folin–Lowry method. Sixty micrograms of protein from each cell treatment was loaded and separated by 12% SDS–PAGE and transferred to nitrocellulose membrane. The membranes were blocked, washed and probed using 1:700 dilution of anti-c-MYC (Invitrogen, catalogue number 710007) and anti-GAPDH antibodies (Invitrogen, catalogue number 398600) for overnight at room temperature. The blots were washed and immunoreactive bands were incubated with a 1:2,000 dilution of respective enzyme conjugated secondary antibodies for 2 h at room temperature. Binding signals were visualized with enzyme substrate (MP) and relative band intensities were determined using ImageJ software.

Exon-specific assay. CA46 cells were treated with Tx 1 at 1 and 2 μM concentration for 24 h. The cells were washed in PBS twice and the RNA was isolated using the trizol kit according to the manufacturer’s protocol (ThermoFisher Scientific, catalogue number 15596081). Reverse transcription of the RNA was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, catalogue number 4308081). The cDNA was amplified in PCR using Power SYBR Green PCR Master Mix (ThermoFisher Scientific, catalogue number 4367659) with the exon 1 and exon 2 forward and reverse primers in Light Cycler 480 II (Roche) system. For exon 1 and exon 2, the relative mRNA expression was normalized by the GAPDH expression. Primers: Exon 1: 5′-CACGGAATTTGCGCATGAC Exon 1 R: 5′-GCAAGAGGCTCCTCCAGAC Exon 2: 5′-CCCTCAAGGATGCCCTC Exon 2 R: 5′-AGGACGCTGATTTTCTTCA

FACS analysis for apoptosis. HCT116 cells were treated with increasing concentrations of Tx 1 for 24 h and collected by trypsinization. Cell pellet was resuspended in 500 μl 1X Annexin V binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, 2.5 mM CaCl₂) and incubated with 5 μl FITC-Annexin V (ThermoFisher Scientific, catalogue number A13199) and 1 μl 100 μg ml⁻¹ propidium iodide (ThermoFisher Scientific, catalogue number P3566) for 15 min. Finally flow cytometry was performed by BD-LSR flow cytometer (BD Biosciences, SanDiego, CA, USA) for apoptosis assay. Approximately 10,000 HCT116 cells were monitored for each sample.

Data availability. The authors declare that all data supporting the findings of this study are available in the article and in Supplementary Information file. Additional informations are available from the corresponding author upon request.

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Author contributions

J.D. and D.P. designed the experiments. D.P. synthesized the compounds and performed HPLC. D.P. and P.S. performed TGS reactions and isolation of the leads. D.P. and P.S. carried out FRET, Fluorescence and CD spectroscopy. P.S. and T.D. performed biological experiments. J.D. supervised the experiments. All authors analysed the data and J.D. and P.S. wrote the manuscript.

Additional information

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